VARIATIONS IN THE PLASMA CHOLESTEROL AND CHOLESTEROL ESTER CONTENT IN HOG CHOLERA

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(Received for publication, October 5, 1929)

Abnormalities in the cholesterol content of the blood have been noted in a number of acute infectious diseases.

Bacmeister and Henes (1) observed that during the stage of hyperpyrexia in acute infectious diseases as e.g. typhoid fever, scarlet fever, pneumonia, erysipelas and sepsis the blood cholesterol content was less than normal. With defervescence the blood cholesterol content approached or rose somewhat above its normal level. On the other hand, Gorter and ten Bokkel Hinnink (2) found that infection of rabbits with a virulent strain of paratyphoid bacilli resulted in a considerable hypercholesterolemia in those cases in which infection was rapidly fatal. In less acute infections, which did not terminate fatally, the hypercholesterolemia gradually disappeared and the cholesterol content became normal without having exhibited subnormal values. Kipp (3) found that in pneumonia the changes in the serum cholesterol content could be divided into three stages; first, a hypocholesterolemia, early in the disease, the degree of which was dependent upon the amount of lung involvement and the intensity of the infection; secondly, a hypercholesterolemia during and continuing for a varying period of time after the resolution of the pneumonic exudate, and lastly, a return to normal with convalescence. Stern (4) observed that in scarlet fever the hypocholesterolemia present at the onset of the infection gradually changed until the values reached the lower limits of normal, where they persisted throughout the latter part of the infection and during convalescence. Malinut and Santner (5) found that in puerperal fever the blood cholesterol level was increased even above the hypercholesterolemia of pregnancy. De Paula Santos (6) and Borel, Pons, Advier, and Guillerm (7) observed that in malaria the cholesterol content of the blood was subnormal. A hypocholesterolemia was noted by Rouzaud, Sucquet, and Cabanis (8) in the secondary stage of syphilis, normal values being found in the primary stage. Marie (9) noted that rabies was ordinarily accompanied by a hypercholesterolemia.

In general, then, most bacterial diseases thus far studied show subnormal blood cholesterol values, paratyphoid infection in rabbits and

puerperal sepsis being apparent exceptions. In the case of the latter as in rabies, a filterable virus disease, there is a hypercholesterolemia. The mechanism of or functions served by the changes observed in the blood cholesterol have never been adequately explained. Some suggestions have, however, been advanced by various investigators.

Leupold and Bogendörfer (12) considered that the hypocholesterolemia observed during acute infections was the result of combination of cholesterol with toxic products of the infectious agent in a protective manner. That such a combination is conceivable is indicated by Ransom's (13) investigations concerning the inhibitory action of cholesterol for saponin hemolysis and Noguchi's (14) concerning its antihemolytic activity against agaricin and tetanolysin. Numerous subsequent investigators have observed other toxic substances that could be neutralized in whole or in part by cholesterol *in vitro*. *In vivo*, the antitoxic action of cholesterol is less evident. Filia (15) who noted that cholesterol, *in vitro*, had a neutralizing action on diphtheria toxin observed that it was completely devoid of action when injected into an animal given a fatal dose of diphtheria toxin. Lubinski and Stern (16) were unable to protect mice and rabbits against dysentery, tetanus or diphtheria toxins by the administration of considerable amounts of cholesterol. Beumer (17) noted that guinea pigs fed large amounts of cholesterol lived longer than controls when given a lethal dose of diphtheria toxin.

The effects on the blood cholesterol content of the injection of toxic substances into animals have been observed. Hueck (18) reported for Köhler that the injection of saponin into animals caused, at first, a reduction in blood cholesterol, followed by an increase. By repeated small doses the blood cholesterol could be maintained above its normal level. Kollert, Kofler, and Susani (19) and Handovsky and Trössel (20) noted only an increase following the administration of saponin to animals. Adler (21) and Yonemura and Fujihara (22) found that intravenously injected bile acids reduced the cholesterol content of the blood. The latter investigators called attention to the fact that certain snake venoms had a similar effect on the blood cholesterol. Leupold and Seisser (23) used, as one of their methods for lowering the blood cholesterol of rabbits, the injection of diphtheria toxin.

Recently experimental work on the filterable virus disease, hog cholera, has been conducted in this laboratory and it seemed of interest to determine the variations in plasma cholesterol and cholesterol ester associated with it. Most of the previous investigations bearing on changes in the blood cholesterol content in acute infectious diseases have had to do with spontaneous disease in human subjects and hence have not been entirely controllable or as complete as is possible with induced infections in animals.

EXPERIMENTAL

Inoculation with hog cholera virus was made either intraperitoneally, intracutaneously, or intracorneally. Blood upon which the normal plasma cholesterol and cholesterol ester values were to be determined was drawn either the day the animals were inoculated with virus, just preceding this inoculation, or on the preceding day. The animals were bled from the artery of the tail into a tube, and heparin, 1 mg. per 5 cc. of blood, was used as the anticoagulant. Bloor's method (10) was used in determining the total plasma cholesterol and Bloor and Knudson's method (11) in determining plasma cholesterol ester.

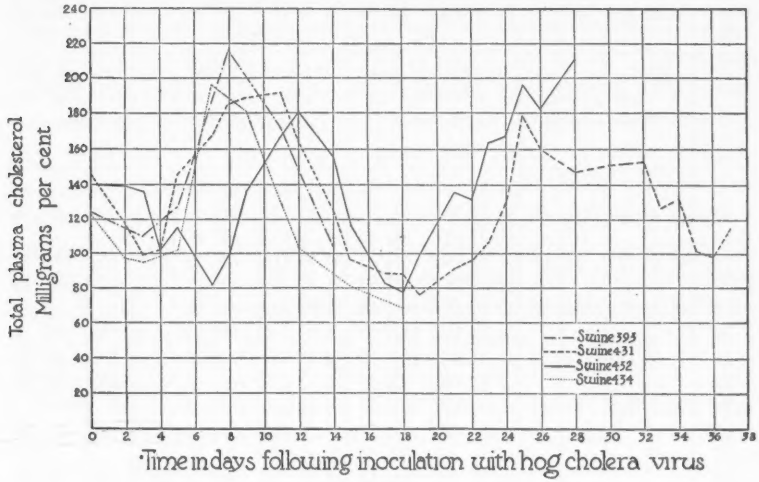

CHART 1

RESULTS

The findings, as regards changes in plasma cholesterol and cholesterol ester, in hog cholera are summarized in Table I and represented graphically in Chart 1. The variations observed were quite uniform for the group of animals as a whole, only slight individual differences being noted. Immediately following inoculation with virus the plasma cholesterol and cholesterol ester content diminished, the hypocholesterolemia persisting in all cases for at least 3 days following inoculation, and in the cases of Swine 432 and 434 for somewhat longer. This interval corresponded to the "incubation" period of the disease and terminated soon after the febrile reaction to the infection

TABLE I

Days after inoculation	Swine 393				Swine 431				Swine 432				Swine 434			
	Temperature	Total plasma cholesterol	Plasma cholesterol ester		Temperature	Total plasma cholesterol	Plasma cholesterol ester		Temperature	Total plasma cholesterol	Plasma cholesterol ester		Temperature	Total plasma cholesterol	Plasma cholesterol ester	
	°C.	mg. per cent	mg. per cent	per cent of total	°C.	mg. per cent	mg. per cent	per cent of total	°C.	mg. per cent	mg. per cent	per cent of total	°C.	mg. per cent	mg. per cent	per cent of total
Normal	38.7	124	70	56	39.0	146	78	53	39.0	140	85	61	38.6	123	74	60
	0.001 cc. hog cholera virus intraperitoneally				0.4 cc. hog cholera virus intracutaneously				0.1 cc. hog cholera virus intracorneally				0.4 cc. hog cholera virus intracutaneously			
2					39.2	117	55	47	39.4	139	92	66	38.9	97	74	76
3	40.4	110	54	49	40.7	99	53	54	39.9	136	88	65	39.5	95	72	76
4					41.5	102	57	56	39.5	102	87	85				
5	41.0	127	83	65	41.6	146	63	43	39.8	115	68	59	40.9	102	65	64
7					41.5	167	92	55	41.8	82	47	57	41.4	196	103	53
8	41.6	216	157	73	42.0	185	104	56	42.0	100	54	54				
9					42.1	189	113	60	41.2	136	69	51	41.6	181	98	54
11	41.0	172	113	66	41.6	192	126	66	42.8	167	134	80	41.2	130	90	69
12					41.0	164	95	58	42.2	181	144	80	41.6	104	61	59
14	40.1	105	57	54	40.4	126	65	52	40.5	156	107	69				
15					40.3	97	57	59	40.1	117	80	68	41.0	81	42	52
17					39.5	89	50	56	39.0	83	50	60				
18					39.5	89	51	57	38.5	78	50	64	40.7	69	35	51
19					38.5	77	54	70	38.6	100	48	48				
21					38.4	92	61	66	37.0	136	72	53				
22					38.8	96	57	58	37.0	132	79	60				
23					39.2	107	73	68	35.8	164	59	36				
24					38.8	130	75	58	36.8	168	51	30				
25					39.2	179	86	48	36.2	196	43	22				
26					38.2	160	93	58	39.5	183	56	31				
28					39.0	148	79	53	38.9	211	92	44				
30					39.3	152	78	51								
31					40.0	153	94	61								
32					39.3	154	83	54								
33					39.6	127	92	72								
34					39.5	132	86	65								
35					40.1	102	89	87								
36					40.1	99	63	64								
37					39.5	115	69	60								

became established. Following the early period of hypocholesterolemia a stage of rather marked hypercholesteremia ensued. This period was coincident with the onset of fever, general malaise, irritability, and loss of appetite. The period of hypercholesterolemia was succeeded by a second stage of hypocholesterolemia more marked as to degree and more prolonged than that during the period immediately following inoculation had been. During this stage all of the general symptoms of the disease had become more marked. Physical depression was as a rule complete, the animals lying in their pens in a torpid condition and not moving unless disturbed. Food was completely refused and very little water was taken. There was marked weakness of the legs; loss of weight was evident; respiration was more rapid and noisy; purple splotching of the skin was appearing; and the fever was beginning to diminish. While in this condition Swine 393 and 434 became moribund and were killed for pathological material. Swine 396 was killed for pathological material before it reached this stage. Swine 431 and 432, animals with a less acute type of cholera than the others, showed a second period of hypercholesterolemia. This persisted in Swine 432 until its death and in Swine 431 was maintained for over a week and then was succeeded by a third period of a rather fluctuating hypocholesterolemia. The animals during the last period of hypercholesterolemia had become markedly emaciated, were delirious, unable to stand, with incoordinated movements and a beginning dry gangrene of the ears and areas of the skin. Swine 431 was killed when in this condition for pathological material.

The changes and fluctuations in the plasma cholesterol content in hog cholera were so constant that it seemed of interest to determine the effect of a secondary infection. For this purpose, *Bacillus suisepticus* was chosen since it had been observed that inoculation with this organism was followed very promptly by a marked decrease in the plasma cholesterol content. Swine 396 was inoculated with hog cholera virus and the disease allowed to progress for 7 days through the stage of hypocholesterolemia and into the beginning of the stage of hypercholesterolemia. Then this animal and a normal one, Swine 394, were inoculated subcutaneously with the same culture of *B. suisepticus*. The variations in plasma cholesterol and cholesterol

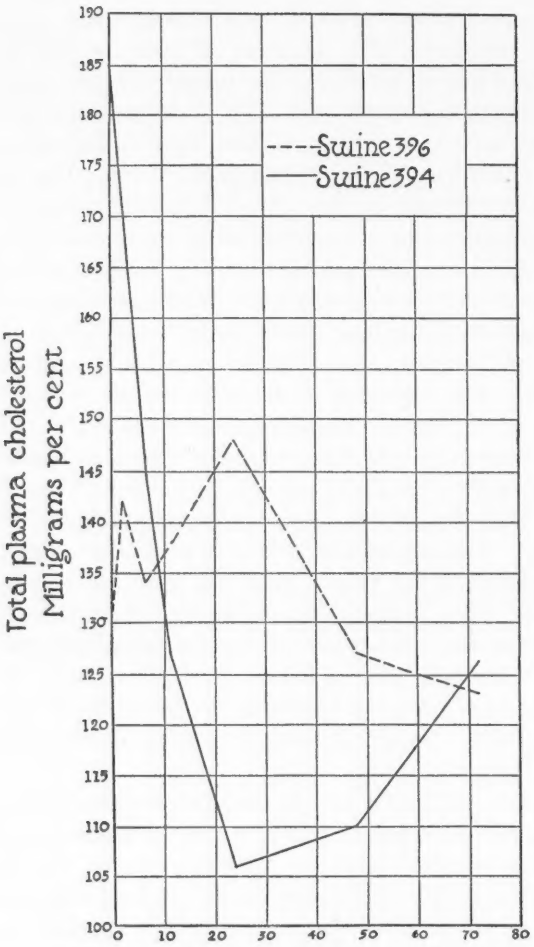
Total plasma cholesterol
Milligrams per cent
190
185
180
175
170
165
160
155
150
145
140
135
130
125
120
115
110
105
100
0
10
20
30
40
50
60
70
80
Swine 396
Swine 394
Time in hours following the subcutaneous inoculation of 5cc. of a 24 hour broth culture of B. suisepticus (376D)

CHART 2

ester observed are given in Table II and represented graphically in Chart 2. The reaction of the cholera-infected animal to infection with *B. suisepticus* was very unlike that of the normal healthy animal. The latter showed a marked and rapidly progressive decrease in plasma cholesterol, the content reaching a low point in 24 hours. The cholera-infected animal, on the other hand, failed completely to show any decrease in plasma cholesterol during the corresponding 24 hour

TABLE II

Time after inoculation		Swine 396				Time after inoculation		Swine 394			
		Temperature	Total plasma cholesterol	Plasma cholesterol ester				Temperature	Total plasma cholesterol	Plasma cholesterol ester	
days	*hrs.*	°C.	*mg. per cent*	*mg. per cent*	*per cent*	*days*	*hrs.*	°C.	*mg. per cent*	*mg. per cent*	*per cent*
Normal		39.2	122	79	65						
0.1 cc. hog cholera virus intraperitoneally											
1		39.4	104	76	73						
2		40.2	111	78	70						
3		41.0	108	28	26						
7		42.0	131	87	66	Normal		39.2	185	91	49
5 cc. of a 24 hour broth culture of *B. suisepticus* (376D) subcutaneously						5 cc. of a 24 hour broth culture of *B. suisepticus* (376D) subcutaneously					
	2	42.2	142	87	61						
	6½	41.6	134	92	69		6½	41.6	144	76	53
	11½		138	83	60		11½	41.4	127	75	59
8		41.1	148	83	56	1		39.5	106	70	66
9		42.0	127	68	54	2		39.0	110	74	67
10		41.8	123	68	55	3		39.0	126	79	63

period. Instead a slight increase occurred which may or may not have been a part of the regular cholera hypercholesterolemia. The plasma cholesterol content of Swine 394 began to return towards the normal sometime after the first 24 hours whereas in Swine 396 the initial slight hypercholesterolemia decreased slightly. Altogether the changes in plasma cholesterol following inoculation with *B. suisepticus* were entirely unlike those taking place in the normal animal.

DISCUSSION

The constancy of the changes in plasma cholesterol and cholesterol ester observed throughout the course of experimentally induced hog cholera suggests a uniformity of mechanism by which the variations are effected. The finding that there is an initial stage of hypocholesterolemia agrees well with observations made by earlier investigators on other acute infectious diseases. The fluctuating plasma cholesterol alterations in hog cholera are unique, however, similar observations having been made in no other acute infection. The significance of these fluctuations is not known. The assumption of previous investigators that in acute infectious diseases variations in blood cholesterol are a manifestation of an antitoxic process, could at most only partially explain the fluctuations observed in hog cholera. These fluctuations may be only an indication of derangement of the physiological mechanism whereby the blood cholesterol level is controlled and be devoid of significance for the clinical course of the disease. The failure of a cholera-infected animal to show a decrease in plasma cholesterol content when inoculated with an organism (*B. suisepticus*) known to be capable of inducing a marked decrease in a normal animal, if it proves to be a regular finding, might be evidence of an inability of the cholesterol regulatory mechanism to function in swine ill with hog cholera.

SUMMARY AND CONCLUSIONS

1. The plasma cholesterol and cholesterol ester content of swine, experimentally infected with hog cholera, exhibit a regular succession of changes. During the period of incubation of the disease, for 3 or more days following inoculation with hog cholera virus, hypocholesterolemia prevails. This is followed by a period of hypercholesterolemia which is coincident with the onset of the clinical manifestations of the disease. The hypercholesterolemia after persisting for from 4 to 7 days, gives way to a second period of hypocholesterolemia more marked and more prolonged than that observed immediately after inoculation. In the experiments of the present work this second period lasted 8 and 11 days in the 2 animals surviving long enough for the study of it and was followed by a second period of

hypercholesterolemia. In the one animal surviving this period for 8 days a third period of irregular and fluctuating hypocholesterolemia set in.

2. A comparison with the results in other acute infections indicates that hog cholera is unique in showing alternating periods of hypocholesterolemia and hypercholesterolemia.

3. A normal hog inoculated with *Bacillus suisepticus* rapidly developed the typical marked hypocholesterolemia whereas an animal infected with hog cholera and then inoculated with *B. suisepticus* failed to show the decrease in plasma cholesterol content.

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ON THE RELATION OF THE ORGANISMS IN THE TUNICA VAGINALIS OF ANIMALS INOCULATED WITH MEXICAN TYPHUS TO RICKETTSIA PROWAZEKI AND TO THE CAUSATIVE AGENT OF THAT DISEASE

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PLATE 5

(Received for publication, October 26, 1929)

INTRODUCTION

In earlier communications (1, 2) one of the present authors has advanced considerable evidence that the small intracellular organism discovered by him in the tunica vaginalis of guinea pigs reacting to the virus of tabardillo represents the specific organism of Mexican typhus. This organism can easily be demonstrated in all animals killed during the early scrotal reaction, whereas it never was met with in numerous normal control animals nor in animals which had recovered from the experimental disease. In its morphology, staining properties and its mode of intracellular multiplication, it exhibits a striking resemblance to Rickettsia prowazeki as found in the gut of lice fed upon typhus patients. The tunica vaginalis regularly proved to be the most infectious tissue, more so than brain, and a positive Weil-Felix reaction can be induced in rats and in rabbits more regularly with tunica than with blood or brain. The same small micro-organism was recently also demonstrated in both of these species. The rat proved to be an even more appropriate animal for the demonstration of this micro-organism than the guinea pig, as was first shown by Maxcy (3). It is frequently found in smears from the tunica of white rats in astonishing numbers within greatly swollen cells from the endothelial covering of the visceral and parietal surfaces. The relation of this organism to typhus fever has been so far cleared up that we were able

to state that it could not be separated etiologically from our strain of Mexican typhus. We still lacked, however, some very important evidence for the identity of the two, namely, the demonstration of the tunica organism in animals inoculated with lice infected with the virus of our strain. Although the findings of Maxcy in a strain of endemic typhus from North Carolina (4) and those of Pinkerton in a strain from Europe (5) have brought full confirmation of the findings of Mooser, the possibility that the tunica organism is a mere secondary invader had not yet been completely excluded. When one considers that the blood serum of a typhus patient regularly agglutinates in high dilutions B. proteus X19 and frequently the bacillus of Plotz, both of which have no etiological relation to that disease, even an organism found with such regularity as the tunica germ must be looked upon with suspicion. Progress can only be expected from experiments with infected lice. The present paper deals with this problem.

Material and Methods

1. Three monkeys have been used in these experiments, Monkeys 1, 2, and 3. As these three animals have not been inoculated at the same time and with the same material, the account of their reaction after inoculation and the result of lice feeding experiments will be presented separately.

2. The strain of body lice used in these experiments was carefully examined for Rickettsia-like organisms before we started. No suspicious organisms could be found in smears from very numerous specimens. The intestinal tract of the great majority proved to be free from demonstrable micro-organisms. The same was true for a number of lice fed for a week on a normal monkey. As the lice did not feed when applied to the monkeys within a Nuttal box, the felt with the lice on it was removed from the boxes each time for feeding and applied directly to the shaved abdomen. They were always closely watched for the whole time of feeding. As a rule they were applied twice a day to an infected monkey and, between feedings, they were kept in an incubator at 32°C.

Experiments with Monkeys

Experiment No. 1.—a. The Reaction of Monkey No. 1. Monkey No. 1 used in this experiment was a young female, a cross-breed between Macacus rhesus and the common macacus. She was inoculated intraperitoneally on May 12th with 5 cc. of blood and 5 cc. of testicular washings (4) from a guinea pig on the third day of fever and scrotal swelling. Examination of smears from the tunica had shown few extra and intracellular organisms. Chart No. 1 illustrates the reaction of this monkey. 120 young lice had been put on her on May 8th.

Twenty-four hours after inoculation the animal's temperature rose to 105 and it was listless and refused all food. Examination revealed that she was menstruating. The next day the fever had dropped and the animal was lively again. Five days after inoculation she was shivering in her cage, was listless again and did not touch any food for nearly two days, when she slowly began to recover, improving steadily despite a continued fever and appearing healthy on the day when the fever dropped to normal. On the second day after defervescence, the lice were transferred to a normal monkey, to avoid the action of the bactericidal power of the convalescent blood in making emulsions of these lice. The lice acted normally until May 26th when large numbers began to die. Before this date only

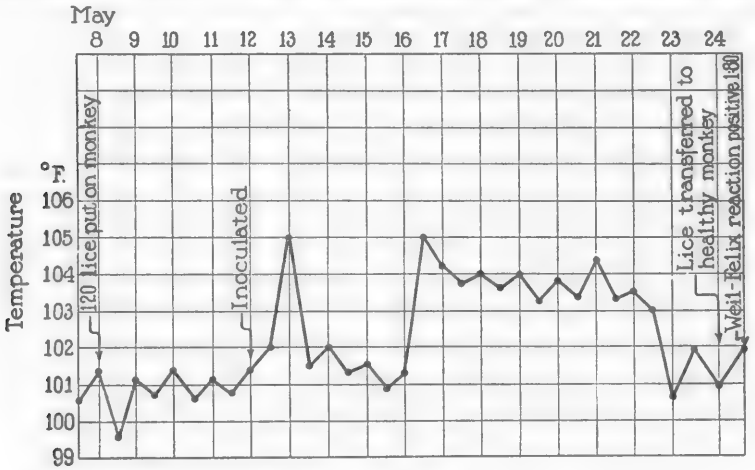

CHART 1. Monkey No. 1

four lice had died, but from then on the number of dead lice increased daily, and on May 30th, only 30 of them were still alive.

b. The Organisms Found in Lice Fed on Monkey No. 1. On May 24th scarce bipolar staining Rickettsiae were found in a dying louse. Every dead and dying louse was from then on examined carefully by making a smear from the dissected intestinal tract. On May 26th a second louse was found to be infected. It contained large numbers of the small red staining diplobacilliform organism described in typhus literature. On May 30th among a large number of dead lice, one was found to be very distended and discolored. It was the only one in which micro-organisms could be found. It contained very large numbers of minute red staining diplobacilli and coccoid forms and slightly larger bacillary forms exhibiting bipolar staining. Smears made from an emulsion of sixteen living lice did not reveal any organisms. Equal parts of this emulsion were inoculated into a

male guinea pig (G1) and a male white rat (Rat 1). The remaining fourteen living lice were then cut longitudinally into halves. From one side a smear was made and the other half was preserved for animal inoculation. In the smears of these fourteen lice Rickettsiae were found in two only. One contained large numbers of the small red staining diplobacillary and coccoid forms whereas the other showed numerous pale bipolar staining bacillary forms. The halves of these fourteen lice were emulsified in a mortar, taken up with 20 cc. of normal salt solution and inoculated intraperitoneally into a male rat (Rat 2) and three male guinea pigs (G2, G3, G4).

c. The Reaction of Guinea Pigs and White Rats Inoculated with Lice Fed upon Monkey No. 1. Nine days after inoculation G4 showed a moderate edema of the scrotum and a temperature of 104.6 F. G2 and G3 had had irregular fever since the time of the inoculation. On the day when G4 had fever and a suspicious reaction of the scrotum, Rat 2 was killed. The examination of its organs revealed a slightly moist peritoneum and there was a fine coat of fibrinous material covering the slightly hyperemic testicles and the parietal surfaces of the tunica. Stained smears made from the surfaces of both testicles and from the tunica parietalis showed numerous heavily infected endothelial cells crowded with reddish staining minute diplobacilli and other cells containing only few slightly larger, bluish staining diplobacilli exhibiting bipolar staining. Both these forms were morphologically and tinctorially indistinguishable from the organisms seen in the lice.

With a saline solution in which both testicles of this rat had been vigorously agitated two guinea pigs were inoculated intraperitoneally. Both developed high fever and a beginning scrotal swelling sixty hours after inoculation and numberous extra and intracellular organisms were found in smears from the tunica of both animals. A separate strain of typhus (louse strain No. 1) was started from one of these animals. This strain exhibited all the characteristics of the passage strain with the exception that in the first two transfers, it showed a remarkably short incubation period and there was an unusually severe general and scrotal reaction.

G4 was killed on the third day of fever. The slight scrotal swelling had completely subsided on the second day. The autopsy was negative except for the testicles which were hyperemic and covered by a fine film of fibrin. Smears made from both sides revealed the typical cytological picture that is found in animals reacting to tabardillo but it was only after a very prolonged search that a few intracellular organisms with characteristic morphology were found within an endothelial cell.

G1, which had been inoculated with an emulsion of sixteen lice, died of pneumonia twelve days after inoculation. No indication of a typhus infection could be found at autopsy.

The slight irregular fever of G2 and G3 lasted for about ten days but there was never any sign of scrotal swelling found in spite of daily examination.

d. Cross-Immunity Tests between the Strain Isolated from Rat No. 2 and the

Original Passage Strain. There was complete cross-immunity between the louse strain No. 1 and the original passage strain. Not only did animals previously inoculated with one of them not show any fever when subsequently inoculated with the other strain, but also the scrotal reaction failed to make its appearance and no tunica organisms could be found in these animals. Guinea pigs 2 and 3, however, reacted typically after inoculation with the passage virus, and the tunica organism could easily be demonstrated in smears from the processus vaginalis. Aside from the lesions mentioned, these two animals showed evidence of a healing diffuse peritonitis.

While there can not be any doubt that the virus of typhus was recovered from a rat inoculated with an emulsion of lice infected with Rickettsia prowazeki, the failure of two guinea pigs to react after inoculation with an equal part of the same emulsion was difficult to explain. The circumstance that the organisms in the tunica of another animal (G4) were found only after a very prolonged search, however, was not surprising as the animal was killed on the third day of fever when occasionally extremely few or no infected cells may be found in the tunica of animals reacting to tabardillo. Two previous observations may furnish an explanation for the failure of G2 and G3 to become infected. When the present strain of tabardillo was isolated nearly two years ago (6), only one of two guinea pigs inoculated with the same amount of blood of a case of Mexican typhus reacted, whereas the other not only did not show any evidence of disease, but reacted typically when inoculated later with the passage virus. More frequent was the observation that the virus does not take easily in an animal whose peritoneal cavity is not free from another kind of infection. The reaction of the tunica constitutes a primary lesion due to the intraperitoneal inoculation which brings the virus in contact with the endothelial lining of the tunica vaginalis. Acute inflammatory processes within the peritoneal cavity seem to be decidedly unfavorable for the primary localization of the virus within the endothelial cells of the peritoneum. The failure of G1 to react is not astonishing as we had not found any Rickettsia-like organisms in the emulsion from the sixteen lice with which it was inoculated. Rat 1, which was inoculated simultaneously, did not agglutinate proteus X19 a week and 12 days respectively after inoculation. It was concluded therefore that none of the sixteen lice inoculated into Rat 1 and G1 contained the virus of typhus.

Experiment No. 2.—Monkey No. 2, a Macacus rhesus, was a large adult female. She was inoculated intraperitoneally on June 25th with 10 cc. of blood and nearly the entire brain of a guinea pig, killed on the fourth day of fever. At the same time an emulsion made from the scrapings of the surface of both testicles and tunicae parietales was injected, 10 cc. subcutaneously over the abdomen and 10 cc. intraperitoneally. The scrotal swelling of this guinea pig had already begun to subside when it was killed. The careful examination of six smears from both sides revealed a single endothelial cell containing the organisms. It was for this reason that we have inoculated nearly the entire brain and 10 cc. of blood in addition to the tunica emulsion.

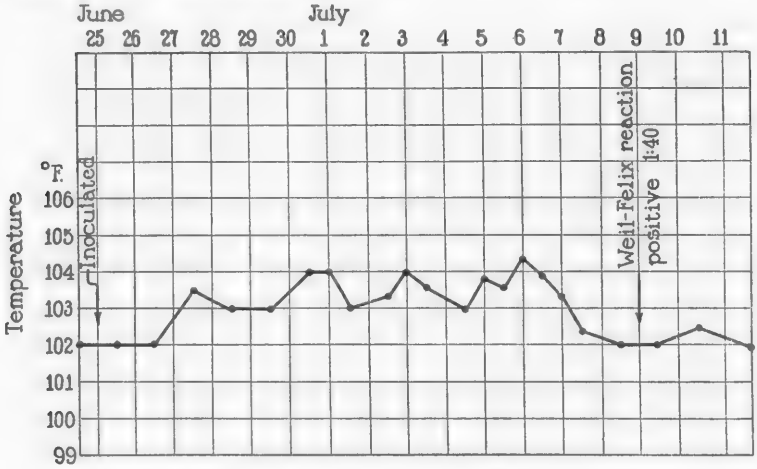

CHART 2. Monkey No. 2

Almost immediately after inoculation, this monkey showed signs of illness. She did not take any food for two days subsequently and remained crouched in a corner of her cage. On the third day, the place where the subcutaneous inoculation had been given became edematous and slightly inflamed. This reaction subsided completely within three days. The temperature of this monkey is illustrated in Chart 2.

350 lice, mostly nymphs, were put on this monkey on the day she was inoculated. They were allowed to feed twice daily as in Experiment No. 1. From the sixth day on, after they had begun to feed on this monkey, two or three of them were killed each day and examined for Rickettsiae. None were found. As the fever of Monkey No. 2 was very mild, we inoculated Monkey No. 3 on July 3rd and transferred the lice to the latter animal when it began to have fever. This was done on July 6th, eleven days after the lice had been put on Monkey No. 2.

This experiment was unsatisfactory and therefore not conclusive for the reason that we interrupted the louse feeding experiment too early. There is no doubt in our minds that the monkey had a mild attack of tabardillo on account of the typical local reaction (7) and a positive Weil-Felix reaction two weeks after inoculation. We transferred the lice to another monkey in order to take full advantage of the large crop of young lice carefully reared for this experiment. We were afraid that by feeding the lice unsuccessfully for a longer time on Monkey No. 2, they might have become too old to be used in another experiment, because adult lice live only about 25 to 30 days. That the lice had not become infected by feeding on this monkey and that our precaution was fully justified can be seen from the fact that Rickettsiae did not appear in them before the seventh day after feeding on Monkey No. 3, eighteen days after Monkey No. 2 had started to have fever.

Experiment No. 3.—a. The Reaction of Monkey No. 3. Monkey No. 3, a young male, was a cross-breed between Macacus rhesus and the common macacus. He was inoculated with an emulsion from both tunicas and scrapings from the surface of both testicles. This material was taken from a guinea pig on the third day of a very severe febrile reaction and on the second day of a very pronounced scrotal swelling. Part of the emulsion was inoculated intraperitoneally, the rest was introduced beneath the skin of the abdomen, infiltrating a large area of subcutaneous tissue. The examination of several smears from both tunicas before inoculation had revealed exceptionally numerous extra and intracellular organisms in every field of the oil immersion lens. Two guinea pigs inoculated with brain and one with a small amount of the tunica emulsion from the same animal were used as controls. Chart No. 3 gives the reaction of Monkey No. 3. All three guinea pigs developed high fever and a scrotal swelling 60 hours after inoculation. The reaction of the guinea pig inoculated with tunica was especially severe. Immediately after inoculation this monkey was an entirely changed animal. A lively and very friendly pet before, he acted very sick from the start, refusing all food for three entire days, sitting with a painful expression on his face in a corner of his cage, never budging an inch. On handling, he acted as if he had severe pain in his abdomen where the inoculation had been performed. 48 hours after inoculation an extensive inflammatory edema made its appearance at the place of injection. The central part of it became diffusely hemorrhagic the next day. This local reaction lasted for four days. Simultaneously with the swelling the fever started in this monkey. On the second day of fever, the lice were transferred from Monkey No. 2 to this monkey. They were fed upon him twice daily until the temperature had dropped to normal, when they were transferred to a

fresh monkey. Care was taken to feed the lice on the place of the local reaction as experiments with guinea pigs had shown that multiplication of the virus takes place in the skin after subcutaneous inoculation of material from the tunica. Monkey No. 3 had the severest reaction of all three animals. When the fever rose to 106 on July 9th a pronounced injection of the conjunctivae especially over the sclerae appeared. It exhibited a remarkable resemblance in respect to color and general appearance to the conjunctival reaction of human patients suffering from typhus. It persisted until the day on which the fever began to subside. At that time the monkey began to improve rapidly, returning to its normal condition within a few days.

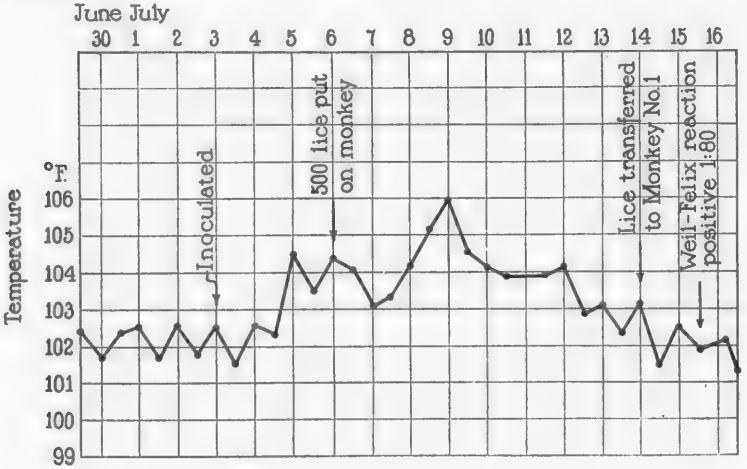

CHART 3. Monkey No. 3

b. The Organisms Appearing in Lice Fed on Monkey No. 3. The lice continued to feed eagerly on this monkey during the entire course of its fever with only an insignificant mortality of three or four lice daily. All lice which were found dead on opening the box were examined microscopically and also those which looked bad and refused to feed, one or two daily. No organisms were found until the seventh day after feeding on Monkey No. 3 when numerous small bipolar staining bacilli were found in teased preparations from the gut of a dying louse and from the guts of two dead lice. It was not until the tenth day that large numbers of the small red staining diplobacillary and minute coccoid forms made their appearance. The lice began to die now in great numbers and these small typical Rickettsiae were found in nearly all lice examined.

Three days after the lice had been transferred to a normal animal or twelve days after they had been put on Monkey No. 3, the box contained only 120 lice.

Sixteen were dead, 44 were dying and sixty fed normally, although the majority of them was distended and somewhat discolored. Smears made from ten lice taken at random on that day showed large numbers of the small red staining Rickettsiae in eight of them. A smear made from a greatly distended and discolored louse showed enormous numbers of these small micro-organisms.

c. The Reaction of Animals Inoculated with Lice Fed on Monkey No. 3. Fourteen rats and four guinea pigs were inoculated with emulsions of lice fed on Monkey No. 3. The lice were put into 95 per cent alcohol for five minutes. They were then transferred to a sterile Petri dish until thoroughly dry before making the emulsion.

The inoculations were made as follows:

July 13, 7th day after feeding: 7 lice into 2 rats (Rat 1 and Rat 2).
July 14, 16 lice into 3 rats (Rats 3, 4 and 5).
July 15, 25 " " 2 " (" 6 and 7).
July 16, 16 " " 2 " (" 8 and 9).
July 17, 14 " " 2 " (" 10 and 11) and 2 guinea pigs (G1 and G2).
July 18, 60 " " 2 " (" 12 and 13) and 2 " " (G3 and G4), and 30 lice separately into 1 rat (Rat 14).

The remaining twenty lice were fixed in Regaud's fluid for histological examination. Prior to every inoculation, a smear was made from each emulsion in order to look for Rickettsiae. This smear was made before the emulsion was taken up in saline solution. Rickettsiae were found in increasing numbers from July 13th to July 18th.

All four guinea pigs (G1, G2, G3, G4) showed fever 60 hours after inoculation and on the end of the third day a pronounced scrotal swelling developed in three of them (Figure 1) whereas the third had only a moderate swelling which increased and decreased slightly during the following three days. One animal (G3) was killed on the third day of fever. The autopsy was negative except for the pronounced and typical lesions of testicles and tunica vaginalis. The intracellular organisms were easily demonstrated in smears from both sides. From this animal a separate strain was started, louse strain No. 2. It induced the typical symptoms of tabardillo in guinea pigs and rats.

Seven rats (1, 3, 6, 8, 10, 12, 14) were killed between the 5th and 6th day after their respective inoculations. The organisms were easily demonstrated in the exudate around the testicles in all of them. In two rats (4, 11) killed after the 7th day, no organisms could be found. Although the lesions of the tunica in these three animals looked typical, the examination of smears indicated that the animals had been killed too late for the demonstration of Rickettsiae because the polynuclear leucocytes which accompany the Rickettsiae in the tunica exudate had already been replaced nearly completely by lymphocytes and large mononuclear leucocytes. From one rat which was positive a separate strain of tabardillo was started (louse strain No. 3). It produced the typical scrotal lesion in all guinea

pigs inoculated and smears from their tunicas revealed with regularity the small intracellular organisms.

The remaining five rats (2, 5, 7, 9, 13) were killed by bleeding twelve days after their respective inoculations. A Weil-Felix reaction performed with their blood gave the following results:

Rat 2 : Positive in a dilution of 1:20.
Rat 5 : " " " " 1:20.
Rat 7 : " " " " 1:10.
Rat 9 : " " " " 1:80.
Rat 13: " " " " 1:100.

d. Cross-Immunity Tests between the Two Lice Strains (1 and 2), the Original Passage Strain and a Strain of Endemic Typhus from North Carolina (Maxcy Strain). Several animals which had recovered from a typical reaction to these lice strains failed to react when subsequently inoculated with a heavy dose of passage virus and virus of the Maxcy strain respectively. Animals recovered after inoculation with the original passage strain and with Maxcy's strain proved to be immune to a subsequent inoculation with one of the louse strains. No organisms could be found in the tunica of these reinoculated animals.

The result of this experiment demonstrates more clearly than Experiment No. 1 that the virus of tabardillo which is characterized by the lesions of scrotum and tunica in guinea pigs is able to multiply in the body louse. Moreover, it demonstrates that the small intracellular organism constantly present in the tunica vaginalis of our passage strain can be recovered easily from rats and guinea pigs after the virus has passed through the louse. An observation made during these lice experiments has a special bearing on the significance of the tunica germ. Monkey No. 3 which was inoculated with material containing exceptionally numerous tunica germs had a very severe reaction and nearly all lice which were allowed to feed upon him became infected with Rickettsia prowazeki. Monkey No. 1 which had received blood and washings from a tunica which had shown relatively few organisms had a mild reaction and only a small percentage of lice became infected with Rickettsia prowazeki. Inoculation into guinea pigs and rats revealed that few of these lice contained the virus of typhus. In the tunica which was used to inoculate Monkey No. 2 only very scarce organisms could be demonstrated after a prolonged search. The reaction of this monkey was so mild that only the positive Weil-Felix reaction gave us the security that his mild fever

was really due to typhus. Not a single louse became infected with Rickettsia prowazeki by feeding on this monkey.

The Inoculation of Lice by the Method of Weigl (8)

5 cc. of human blood were drawn from a vein, defibrinated and some sodium citrate added. The testicles of a guinea pig killed within 24 hours after the appearance of the scrotal swelling were vigorously agitated in the blood. The sodium citrate was added in order to prevent coagulation of the fibrinogen which collects around the testicles during the early scrotal reaction. This mixture was inoculated rectally into 40 lice with a capillary glass pipette attached to a rubber bulb. After inoculation the lice were worn continuously by an immune person. Every day two lice were removed from the boxes for microscopical examination. The first Rickettsiae were found in a louse four days after inoculation. They were now found in increasing numbers in every louse examined. After the seventh day their number was enormous and the lice began to die. On the tenth day all lice which still remained in the box were found to be dying. Examination revealed that the epithelial lining of their guts was completely destroyed by Rickettsia prowazeki. This experiment was repeated twice with the same results. Several guinea pigs inoculated intraperitoneally with emulsions from these lice developed the typical symptoms of Mexican typhus and the tunica organism was easily demonstrated in all of them. The peritesticular exudate of three rats killed five days after the inoculation with such a louse emulsion contained enormous numbers of the small intracellular organisms.

If we compare the results of the preceding experiment with those of the feeding experiments we see that Rickettsia prowazeki appeared four days after a rectal inoculation and in 100 per cent of the lice, whereas in lice fed upon infected monkeys Rickettsia prowazeki did not appear until the seventh day and only a certain percentage of the lice became infected. This, however, is not astonishing as the organisms are always easily demonstrated during the early scrotal involvement whereas they never are found in the blood of animals reacting to typhus. More significant is the fact that the exudate around the testicles during the early scrotal reaction invariably contains several thousand infective doses of the virus of typhus. As a rule, the same amount of a tunica emulsion is at least five hundred to a thousand times more infectious than the same amount of blood. The experiments demonstrate therefore that lice inoculated rectally with concentrated virus of typhus became earlier and absolutely regularly infected with Rickettsia prowazeki as compared with lice which had fed on the much less infectious blood.

The following two experiments were carried out with the aim of separating the causative agent of typhus from Rickettsia prowazeki and from the organism in the tunica.

The Influence of Dilution of Tunica Emulsion on the Occurrence of Rickettsia and the Causative Agent of Typhus in Inoculated Lice

A guinea pig was killed at the beginning of the scrotal swelling and both testicles vigorously shaken in 10 cc. of citrate solution. This suspension was arbitrarily called dilution 1:10 although the amount of exudate in the scrotal sacs was far less than 1 cc. Dilutions were then made from this suspension and defibrinated human blood added to each dilution. Three separate groups containing 20 lice each were inoculated rectally, one group with a dilution of 1 to 100, one with a dilution of 1 to 1000, and one with a dilution of 1 to 10,000.

The following results were obtained:

In the first group of lice Rickettsia prowazeki appeared on the seventh day and the lice began to die on the twelfth day after inoculation. In the second and third groups Rickettsia prowazeki appeared on the ninth and twelfth day respectively. The lice of the second group began to die on the thirteenth day and several lice of the third group died on the fifteenth day. Examination of these dead and dying lice revealed large numbers of Rickettsia and the causative agent of typhus was demonstrated in several lice of each group separately by inoculation into guinea pigs. The tunica organism was easily found in these animals.

Sixteen days after inoculation all lice of Groups 1 and 2 had died. Of Group 3, however, eight lice continued to feed. They were killed on the twentieth day by emulsifying them in a few drops of normal saline solution. A loopful was used for making a smear. The rest was taken up with 10 cc. of normal saline solution and inoculated into two guinea pigs. No Rickettsiae could be found in the smear and the guinea pigs remained normal and did not show any immunity when later inoculated with an emulsion of tunica from the passage strain.

This experiment failed to separate Rickettsia prowazeki from the causative agent of typhus. Whereas the dilutions of 1:100 and 1:1000 of the tunica exudate infected all lice with Rickettsia prowazeki this organism appeared only in a small majority of the lice inoculated with a dilution of 1:10,000. Lice in which Rickettsia prowazeki was demonstrated harbored also the causative agent of typhus whereas eight lice of the third group which were free from Rickettsiae did not contain the causative agent of typhus. The amount inoculated into each louse was about 2 mgrs. Since the dilution inoculated into lice of group three was 1:10,000, the amount of tunica exudate received by each of these lice was one five millionth of tunica exudate. If Rickett-

sia prowazeki and the causative agent of typhus were two different organisms, we should expect that the inoculation of such a small quantity of tunica which is just beyond the limit where each dose contains the causative agent or Ricketstia prowazeki should separate them by the help of louse inoculation, given the extremely high susceptibility of the louse for both of them. One could of course, object that not all lice of Group 3 were equally susceptible to Rickettsia prowazeki and that our experiment does not prove that the quantity of tunica exudate inoculated into the eight lice which remained free from organisms did not contain the tunica organism. We are fully aware of this possibility. With such an assumption, however, we would presume that the degree of susceptibility of the lice for two different organisms is exactly alike, and that these two organisms always occur in the same number in an infectious material, and that the presence of one conditions the presence of the other and vice versa.

The Incubation Period of Mexican Typhus and the Time of the Appearance of the Organism in the Tunica Vaginalis

It is commonly thought that a febrile reaction in guinea pigs appearing earlier than six to seven days after inoculation is not due to the virus of typhus. The inoculation of concentrated tunica emulsion prepared from an animal during the early scrotal reaction is followed by fever and swelling of the scrotum within less than seventy-two hours. The Rickettsia-like organisms can regularly be demonstrated in smears from the tunica sixty hours after inoculation and repeatedly we were able to find them already at the end of the second day. We made, therefore, three successive transfers with tunica emulsions each sixty hours after the respective inoculation and injected twenty lice with an emulsion made from the tunica of an animal of the third transfer. This animal was killed fifty hours after it had been inoculated.

Result: All lice became heavily infected with Rickettsia prowazeki within eight days and the virus of typhus was demonstrated in six of them separately by inoculation into guinea pigs. In all six animals the tunica organism was demonstrated.

This experiment demonstrates that the virus of typhus and Rickettsia prowazeki make their appearance simultaneously in the tissue of guinea pigs. The experiment constitutes another fruitless attempt to separate Rickettsia prowazeki from the virus of typhus.

The Organisms in the Lice

The organisms found in our lice have already been referred to in our experiments as Rickettsiae for the sake of brevity and because they corresponded morphologically and tinctorially to the germs described in lice fed upon typhus patients by da Rocha Lima. We wish, however, to emphasize the morphological identity of the organism found in our lice with those found in the tunica of animals inoculated with the passage strain and with the three strains started from lice.

The organisms found in lice killed early after feeding on an infected monkey or after an infective enema were small pale bluish or purplish staining bacilli (with Giemsa solution) which exhibited more or less decided polar staining. As the infection of the lice progressed the minute red or purplish staining diplobacillary and coccoid forms began to show up in increasing numbers whereas the slightly larger bacilliform organisms became scarcer. Exactly the same phenomenon can always be seen in smears from the tunica of rats and guinea pigs provided that these animals are killed at the proper time. In endothelial cells which contain few organisms the larger pale bluish staining bacilli as a rule are present, whereas in heavily infected cells which are distended and about to rupture, the small red staining diplobacillary and coccoid forms are invariably met with. It seems, therefore, that the minute forms liberated from heavily infected cells by disintegration of the latter assume the form of somewhat larger bacilli after they invade a fresh cell of the louse or of an infected mammal. Wolbach, Todd and Palfrey (9) had already described this behavior in lice and our observation in guinea pigs and rats corroborates their findings in this respect. When the larger bacilliform organisms are distributed more or less evenly over the protoplasm of an infected tunica cell, or of an infected epithelial cell of the louse, they may be present in considerable numbers without the admixture of the minute forms. Very heavily infected cells, the protoplasm of which is completely filled with closely packed organisms, contain few or no large forms as a rule. Roundish or irregularly shaped colonies of closely packed organisms within large endothelial cells are also composed almost invariably of the minute red staining diplobacillary forms whereas loosely packed colonies are composed as a rule of the larger bipolar staining organism. Especially after careful differentiat on of the smears with 95 per cent alcohol the bipolar staining can be seen very clearly. At the first glance one would think that the small diplobacillary forms may not be properly recognized as such on account of their great number in which individual organisms can frequently be recognized only with difficulty. This, however, can easily be ruled out by the observation of large numbers of the small forms when found strewn about cells which had ruptured while making the smears or which had already disintegrated spontaneously in situ before the smear was made. It seems, therefore, that the larger bacillary forms develop only as long as there is enough room and perhaps

food within the cell, whereas the minute forms appear when these conditions become less favorable. We got the impression that the larger bipolar form represents the actively multiplying stage of the organism, whereas the small coccoid forms represent a resting stage. The size of a bipolar staining rod corresponds closely to the size of a diploform composed of the two minute coccoid organisms. A transitional form exhibiting hour-glass shape can frequently be observed. Arkwright and Bacot (10) and also Wolbach, Todd and Palfrey (9) described long bacillary and filamentous forms which showed indications of the formation of the small forms within their body. Similar pictures could occasionally be seen in the tunica cells of our guinea pigs and rats although much shorter ones than those mentioned by these investigators. The very large coiled forms described by other investigators in the gut of lice have never been seen by us in the tunica nor did we find them in sections or smears of lice.

GENERAL DISCUSSION

The result of these experiments is clear cut. Body lice fed upon monkeys inoculated with a strain of Mexican typhus carried along in guinea pigs for nearly two years became infected with the organism known as Rickettsia prowazeki. The inoculation of such lice into guinea pigs and rats induced in the latter animals the typical symptoms of tabardillo. The small intracellular organism found first by Mooser (1, 2) in guinea pigs inoculated with Mexican typhus and later by Pinkerton (5) in a strain of typhus from Europe and by Maxcy (3, 4) in guinea pigs and rats inoculated with endemic typhus from North Carolina could easily be demonstrated in animals inoculated with an emulsion of lice fed upon monkeys inoculated with Mexican typhus. The same results were regularly obtained with lice inoculated with an emulsion of tunica vaginalis by the method of Weigl (8).

Whereas up to the time of the conclusion of these experiments, we were only able to state that the organism found in the tunica vaginalis was constantly associated with our strain of Mexican typhus and that in its morphology and tinctorial behavior it is indistinguishable from Rickettsia prowazeki in lice, we know now that it accompanies the causative agent of typhus from the passage strain via monkey through the louse back into the tunica of rats and guinea pigs where it multiplies again exclusively within cells as it does in the louse, in other words, that the intracellular organism found in the tunica vaginalis of our animals is identical with Rickettsia prowazeki. The present experiments do not advance any startling new knowledge concerning

the etiology and pathology of typhus. They show again, however, and more impressively than had been demonstrated before that the causative agent of typhus cannot be separated from Rickettsia prowazeki. The causative agent of typhus seems to exist in the louse and in the mammal in one form only, namely, in the form of Rickettsia prowazeki. Even this is not an entirely new finding. Kuczynski (11) and especially Wolbach, Todd and Palfrey (9) had already advanced considerable evidence that the causative agent of typhus is present in man and in animals in the form of Rickettsia prowazeki. That their conclusions have not been universally accepted is due to the uncertainty of their method of demonstrating Rickettsia prowazeki in sections of mammalian tissue. This can clearly be seen from the circumstance that Wolbach, Todd and Palfrey (8) consider globular massing within endothelial cells as the most characteristic behavior of Rickettsia prowazeki in mammalian tissue and form their finding of Rickettsia prowazeki within the endothelial leucocytes which constitute the nodular lesion of typhus. Such globular massing can also be seen in smears from the tunica, but it represents only a transitional stage of the intracellular multiplication. The most characteristic finding in mammalian tissue is the occupation of the whole protoplasm of an infected cell by enormous numbers of closely packed organisms which cause great distention of the invaded cell and finally lead to its disintegration with liberation of the intracellular germs, exactly as is seen in the case of Rickettsia prowazeki in the louse. The same course of events can also be demonstrated within Regaud fixed tissue of guinea pigs after staining with Giemsa solution.

As long as the organisms are multiplying within an infected cell the surrounding tissue remains quiescent (12). As soon, however, as the organisms are liberated by disintegration of an infected cell, a sudden acute inflammatory reaction flares up around the disintegrating cell. The Rickettsiae which are not evacuated into the blood stream or which do not gain entrance into a new endothelial cell are taken up by polynucular leucocytes, where they are digested. The leucocytes in turn are taken up by large mononucular leucocytes which gather concentrically around them, giving rise to the typical nodule of the typhus literature. This nodule is not an early lesion but represents a healing stage of a typhus lesion and never contains any demonstrable Rickettsiae.

The demonstration that Rickettsia prowazeki multiplies within infected endothelial cells of the mammal exactly as it does in the cells of the stomach of the louse, causing great distension and finally disintegration of the invaded cells, constitutes alone strong evidence for the specificity of this organism in typhus.

All attempts to separate the causative agent of typhus from the tunica organism or Rickettsia prowazeki have failed completely. The following evidences have so far accumulated in respect to the specificity of Rickettsia prowazeki in typhus: Rickettsia prowazeki is only found in lice fed upon typhus patients (da Rocha Lima (13)). Its presence in lice is constantly associated with the virus of typhus (Wolbach, Todd and Palfrey (9)). Lice inoculated rectally with any material containing the virus of typhus invariably become infected with Rickettsia prowazeki and simultaneously there is a pronounced multiplication of the virus of typhus (Weigl (8)). Emulsions of Rickettsia prowazeki made from lice are agglutinated by serums of cases of typhus (Otto (14)) and also by serums of guinea pigs reacting to typhus which never give a positive Weil-Felix reaction (Weigl (8)). Organisms morphologically simulating Rickettsia prowazeki were demonstrated in sections of specific lesions of typhus (Kuczynski (11), Wolbach, Todd and Palfrey (9)). Recently the same organism was regularly demonstrated in smears from the tunica vaginalis of guinea pigs reacting to a strain of Mexican typhus (Mooser (2)). Pinkerton (5) found the same organism in a strain of European typhus, and Maxcy (4) in a strain of endemic typhus from this country. At this occasion it is well to remember that the virus of typhus is held back by bacterial filters (Anderson and Goldberger (15), Ricketts and Wilder (16), Olitsky (17)). The size of the specific organism must therefore lie within the range of microscopic visibility. Since in an infected louse only Rickettsia prowazeki can be found and since the same holds true for the tunica vaginalis, it is almost impossible to elude the conclusion that Rickettsia prowazeki and the causative agent of typhus are identical. All physical and chemical influences which kill the specific agent invariably kill also Rickettsia prowazeki. Furthermore, we have shown that the incubation period for both is the same in guinea pigs. Since the lesions in the tunica vaginalis show all the histologic characteristics of an uncomplicated typhus infection

(2) and since these lesions regularly contain no other organisms but Rickettsia prowazeki, we have to conclude that the latter organism is etiologically responsible for them. If we question therefore, the specificity of Rickettsia prowazeki in typhus, then we have also to question the specificity of the histopathology of that disease.

SUMMARY

Healthy lice became infected with Rickettsia prowazeki after feeding on monkeys inoculated with a strain of Mexican typhus. The same result was obtained in 100 per cent of lice by rectal inoculation of an emulsion of tunica vaginalis of guinea pigs reacting to the same strain. In the tunica vaginalis of guinea pigs and rats inoculated intraperitoneally with an emulsion of lice containing Rickettsia prowazeki the intracellular organism constantly associated with the passage strain appeared regularly. Rickettsia prowazeki found in lice and the organism constantly present in the tunica of guinea pigs and rats reacting to our strain of tabardillo are indistinguishable morphologically and tinctorially and their mode of intracellular multiplication is alike in every respect. It is concluded that they are identical. This organism is constantly associated with the causative agent of Mexican typhus, both in mammals and in lice, and all of our attempts to separate them have failed.

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EXPLANATION OF PLATE 5

FIG. 1. Scrotal swelling of a guinea pig (G4, Exp. 3) inoculated with an emulsion of lice fed on Monkey No. 3. The scrotal swelling in guinea pigs is characteristic of Mexican typhus.

FIG. 1

(Mooser and Dummer: Mexican typhus and Rickettsia prowazeki)

THE RECOVERY OF VACCINE VIRUS AFTER NEUTRALIZATION WITH IMMUNE SERUM

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(Received for publication, November 27, 1929)

It has been shown by Todd (1) and by Andrewes (2) that mixtures of a virus, e.g., vaccine virus with its specific antiserum which are without effect when introduced into the skin of a rabbit, become lesion-producing if diluted with Ringer's solution. Prior to the observation of this phenomenon, it was generally considered that if a virus is placed in contact with its specific immune serum *in vitro*, either a destruction of the virus takes place or union occurs between the two with loss of infectivity—so-called neutralization of the virus. The discovery of the dilution effect has completely changed current conceptions of the phenomenon.

The nature of antigen-antibody reactions in general is still unsolved. Ehrlich (3) believed that the toxin-antitoxin reaction, resembling in certain features that of the virus-immune serum mixtures, is analogous to the interaction of a strong acid and a strong base, which is complete in one direction and only imperfectly reversible. Arrhenius and Madsen (4) are opposed to this conception and regard the toxin-antitoxin reaction as resembling that of a weak acid and a weak base, leading to an equilibrium when appreciable amounts of both reacting substances and neutral salt are present. Bordet (5) disagrees with both and looks upon the toxin-antitoxin reaction as an adsorption phenomenon in which the antitoxin is distributed equally upon all of the toxin molecules. Still less is known about the nature of the immunological reaction of virus and specific immune serum than about the toxin-antitoxin neutralization.

The purpose of the present paper is to record certain observations made upon neutral mixtures of vaccine virus and its immune serum under various conditions.[1]

[1] We wish to thank Dr. D. A. MacInnes of The Rockefeller Institute for some useful suggestions.

EXPERIMENTAL

Source of Virus. The vaccine virus consisted of the Levaditi (6) strain of neurovaccine, propagated in rabbits' testicles after the method of Noguchi (7). The testicles of normal rabbits were inoculated with a saline suspension of this virus and two or three days later, when they exhibited marked orchitis, the animals were etherized, exsanguinated, and the organs removed under aseptic conditions. The testicles were weighed, minced, and ground in a mortar with a small amount of sand, to which Ringer's solution or distilled water was added to make a 10 per cent suspension. The suspension was centrifuged at a low speed for a minute and the supernatant fluid decanted. The fluid was arbitrarily designated a 1:10 dilution of the virus. The procedure of preparation of the suspension was carried out under under sterile conditions.

Immune Serum. This was obtained from rabbits which had recovered from characteristic experimental vaccinia. The serum was procured, pooled, and bottled without preservative, using aseptic precautions. It was not inactivated and was kept at a temperature of about 4°C.

Test Animals. Healthy, full-grown, white rabbits were used in all tests. The hair was removed from the flanks by careful shaving or by barium sulphide, preferably by shaving. The inoculations, with few exceptions, were made intracutaneously. If care is used, circular lesions can be obtained with the endermic technique. The lesions were measured up to three days after their appearance, at which time secondary lesions generally occurred.

Preparation of Neutral Mixtures. Neutral mixtures of vaccine virus and its immune serum were usually prepared by adding to a given amount of antiserum an equal quantity of a 1:200 or 1:400 dilution of suspension of fresh virus. No incubation was required, since neutralization occurred instantly.

Experimental Results

Cataphoresis Tests

Our intention in performing cataphoresis tests on neutral virus-immune serum mixtures was to note whether the virus could be separated from its antiserum by means of an electric current. A typical experiment, which on repetition gave similar results, is shown in Protocol I.

Protocol I. The method of cataphoresis as applied here has already been fully described (8). As preliminary tests, we studied by means of cataphoresis vaccine-virus infected testicles of rabbits in suspensions at pH = 6 to 8. The virus was uniformly recovered at the anode. The prior observations of Douglas and Smith (9) that the virus, or material containing the virus, migrates in an electrical field and carries an electronegative charge was thus confirmed.

There were prepared 100 cc. of equal parts of virus and antiserum at a pH = 7.8, which was proved ineffective by animal inoculation test. After three hours' cataphoresis at 4.8 milliamperes with a potential drop of 117 volts, active virus was obtained at the anode, as revealed by intracutaneous inoculation of anodic material in the rabbit.

In repeated experiments, the pH of the neutral mixtures of virus and immune serum measured, respectively, 6, 7.5, 7.6, and 8; the milliamperage varied from 3 to 4.5; the potential drop from 115 to 118 volts (or from 38.3 to 39.3 volts per tube) and time constantly three hours. In all these tests the virus was again revealed at the anode.

The results obtained from repeated tests show that from mixtures which are ineffective as such, active virus can be recovered from anodic material after cataphoresis. Hence it can be inferred that in neutral mixtures of the virus and its antiserum, the former is not destroyed by the serum.

Influence of Electrical Charge on Union

Bedson and Bland (10) have recently reported that they have been able to determine the electrical charge of herpes and vaccinia virus particles by measuring the ascent of the particles into negatively charged blotting paper dipped into suspensions of the viruses for given periods of time. It was thought that a change in the electrical charge of the vaccine virus particles might be demonstrated by this method, if union occurred with its specific antiserum.

An example of the results obtained in five separate experiments is given in Protocol II.

Protocol II. 15 cc. of a 1:200 dilution of fresh testicular neurovaccine virus in phosphate buffer at pH = 7.5 were prepared. To this was added 15 cc. of the serum from a rabbit recovered from neurovaccine-virus infection. The mixture was divided into two portions and in each was suspended to the depth of 0.5 cm. a strip of blotting paper 1 cm. wide and 10 cm. long. After one hour the strips were removed and cut into centimeter lengths. The paper corresponding to the third, fourth, and fifth centimeter was used for animal test. In the case of the first strip of blotting paper, the different lengths were applied directly to scarified areas on the flank of Rabbit A, and then rubbed into these areas. In the instance of the second strip of blotting paper, the different lengths were ground with 3 cc. of Ringer's solution, and 0.5 cc. of the supernatant fluid was injected intracutaneously into the flank of Rabbit B. Rabbit A was unaffected, but Rabbit B developed characteristic vaccinal lesions at the sites of inoculation. For controls in each experiment, virus by itself and virus plus normal rabbit serum were used.

From these tests, which, on repetition, gave similar results, it follows that at a hydrogen ion concentration of 7.5, the neutralization of vaccine virus by its specific antiserum does not result in an appreciable change in the electrical charge carried by the virus particles.

Leucocytic Test for Union

Recently Smith (11) has shown that vaccine virus, injected into the circulation of a completely immune rabbit, disappears rapidly, and that the removal from the circulation is a consequence of the fixation of the virus by leucocytes. This test was repeated in a rabbit immune to vaccinal infection, as revealed by its resistance to dermal and corneal inoculation of the virus.

5 cc. of a 1:50 saline suspension dilution of virus were injected into the margin a ear vein. Three hours later the animal was bled from the heart[2] to the extent of 40 cc. The blood was mixed with 4 cc. of 20 per cent sodium citrate to prevent clotting and after centrifugation, the white cells were collected from the buffy layer, washed several times in warm physiological saline solution, and a thick suspension injected endermically into a rabbit. The whole blood, washed red cells, plasma, diluted whole blood, and diluted plasma were used as controls. The latter produced no lesions but the white blood cells induced within forty-eight hours at the site of inoculation a characteristic vaccinal lesion.

Thus this test confirmed Smith's observation in indicating that in an immune animal the virus is removed from the circulation by the leucocytes and, therefore, is not found to be inactivated in the presence of immune plasma.

Effect of Storage in the Cold on Union

It was observed that now and then certain ineffective mixtures of virus and its immune serum, kept at a temperature of about 4°C. for periods of from one to eight weeks, again became potent. In one of five such occurrences, among twenty-seven neutral virus-immune serum mixtures, activity was revealed in a dilution of 1:1 million, even though the mixture had been kept in the icebox for four weeks. The original titration of the virus showed potency in a dilution of 1:10 million.

Although the results are not constant, their frequency of occurrence attracts attention to the fact that the union between virus and its antiserum, if it exists at all, is a loose one.

[2] All operative procedures on animals were made with the aid of ether anesthesia.

Dilution Tests

A series of twenty-seven tests was made to determine the effect of dilution upon neutral mixtures of vaccine virus and immune serum. The results can be summarized as follows:

Protocol III. Methods. Serial dilutions of the mixtures were prepared ranging from 1:20 upwards but, in most instances not carried beyond 1:160, since it

TABLE 1

Size of Lesions on First, Second, and Third Days

Amount injected (cc.)	Dilution of immune-serum			
	1:20	1:40	1:80	1:160
	Size of lesion (mm.)			
First day				
0.05	3	0	6	10
0.1	4	6	8	12
0.2	6	8	9.5	13
0.4	6	8	12	21
Second day				
0.05	6	6	11	15
0.1	8	12	13	17
0.2	7	14	15	17
0.4	12	16	18	22
Third day				
0.05	6	6	10	14
0.1	9	12	14	14
0.2	7	14	16	22
0.4	14	16	20	28

was found that beyond this dilution no visible evidence was apparent of any influence of the serum upon the size of the lesion. The diluents used were physiological salt solution, Ringer's solution, Locke's solution, sterile broth, normal serum, whole blood, and distilled water. The range of pH extended from 5.5 to 8.4.

As a rule, 0.5 cc. of each dilution was injected along a line in the shaved flank of a rabbit. A titration of the virus was always made on the opposite flank. With precision in the technique, the endermic inoculation yields almost always circular lesions.

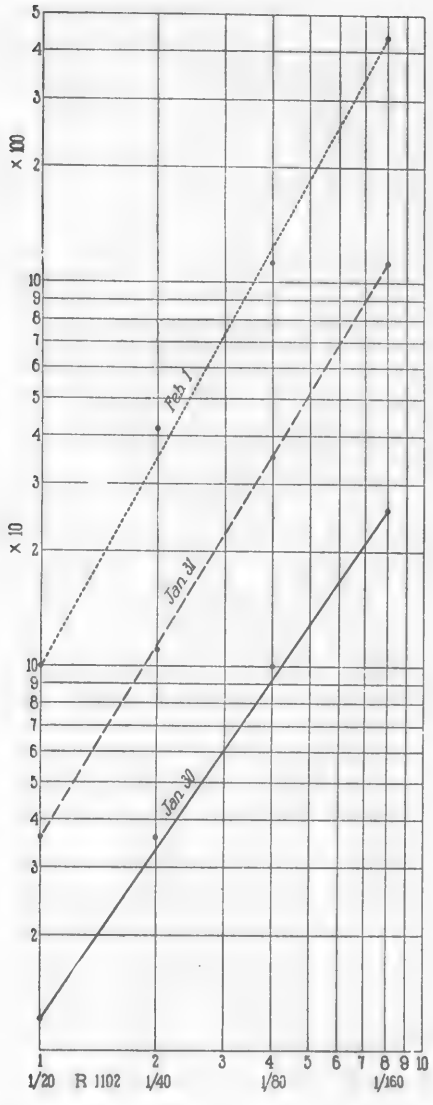

CHART I. Showing the quantitative relations of dilution and size of lesions in logarithmic curves.

It soon became apparent from the varying size of the lesions that, up to a dilution of 1:160, a definite relation existed between the diameter of the lesions and the dilution of the immune serum, since vesicles of the same size could usually be obtained by diluting neutral mixtures beyond 1:160, at which point the immune serum ceased to exert any influence upon the size of the lesion. A typical example is given in Experiment A.

Experiment A. Dilutions of neutral mixtures of vaccine virus and its antiserum were prepared in such a manner that while the concentration of the serum varied in the different dilutions, the concentration of the virus remained constant. 0.05, 0.1, 0.2, and 0.4 cc. of each dilution was injected into the shaved flank of Rabbit A and with the appearance of the lesions measurements of their diameters were made until the secondary eruption appeared. These measurements are given in Table 1.

In Chart I is given the remarkable quantitative relations between dilutions of a mixture of virus and its antiserum and the size of the lesions, in the case of another rabbit. The abscissae are proportional to the dilution of the mixture; the ordinates are proportional to the areas of the lesions.

It was found that if total amounts of diluted mixtures were inoculated, the lesions varied in size in proportion to the dilution of the antiserum, the virus being kept constant in amount at each injection by diluting 0.01 cc. of a neutral mixture with 0.09 cc., 0.19 cc., 0.39 cc., and 0.79 cc. of distilled water respectively. As a control, 0.01 cc. of virus suspension was diluted with an equal amount of distilled water. The total volumes were then inoculated into the flanks of rabbits.

Over-Neutralized Mixtures. When over-neutralized mixtures comprising one part of virus plus 2, 3, or 4 parts of immune serum, which was still in the range of effectiveness, were diluted with appropriate amounts of distilled water, the same dilution effect was observed. However, with these mixtures the dilution had to be increased in proportion to the degree of over-neutralization before a characteristic result could be obtained. For example, 1:80 of a mixture over-neutralized four times produced a certain sized lesion, but 1:20 of a mixture exactly neutralized, and in which the same materials were used, yielded the same sized lesion.

It would appear from these experiments that the size of the lesion bears a direct relationship to the dilution employed. Furthermore, it is possible to estimate the probable size of a lesion which may be induced by diluted mixtures of virus and its antiserum, if given the size produced by a known dilution.

DISCUSSION

Early in the course of the experimental work presented here, it was thought that the effects produced by dilution of mixtures of vaccine

virus and its specific antiserum probably follow the law of mass action (12). Later, with more experimental data at hand, it became apparent that while the observed phenomena might be interpreted as following this law, no conclusive evidence could be noted of union occurring between the virus and its immune serum. This might well, however, be due to very weak combination between the two components, the compound being at all dilutions in equilibrium with varying amounts of its constituents.

The results of cataphoresis experiments demonstrate that the virus can migrate in a neutral, ineffective mixture of virus and its antiserum in a similar manner as in a saline or aqueous suspension. Furthermore, when the virus particles are in contact with antiserum, there appears to be no change in its electrical charge. In addition, if virus is introduced into the circulation of an immune rabbit, it is apparently taken up by the leucocytes and is not found to be in combination with the blood serum. While this method of protection of the animal, first observed by Smith, argues against a conception of stable union between the virus and immune serum, it cannot be, by itself, regarded as conclusive evidence against a loose combination with an equilibrium such as is described above.

The tests show definitely that the dilution of a neutral mixture of vaccine virus and immune serum renders it lesion-producing and that the size of the lesions bears a direct relationship to the amount of diluent employed. The readiest explanation of this striking effect is that given above, that there is a loose combination between the virus and serum, the separation of which follows relations which can be deduced from the law of mass action.

CONCLUSIONS

1. When vaccine virus and its specific antiserum are brought together, no evidence of stable union between them can be determined by the experimental methods employed.

2. A definite relationship exists between the degree of dilution of a neutral vaccine virus-antiserum mixture and the size of the lesion produced by endermic inoculation of rabbits of any given dilution.

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THE EPIDEMIOLOGY OF FOWL CHOLERA

Experimental Studies

I. Introduction

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(Received for publication, October 21, 1929)

The following studies on experimental epidemiology carried out during the past few years have been concerned chiefly with the disease fowl cholera. A report of this work is presented in detail in the succeeding papers (1 a–d); in this introduction, a brief statement will be made of the purpose and scope of the work, the methods employed, and results and conclusions obtained.

The investigation was undertaken primarily to determine whether bacterial infections in general spread according to one and the same type of mechanism. It had been found that in communities of mice or rabbits in which certain intestinal and respiratory infections prevail, epidemics occur in response to a characteristic sequence of events, disturbing the balance between the resistance of the community and the quantity of available microbes (1 e). The steps in the evolution of such epidemics appear to be as follows: first, some influence can be demonstrated in these communities which operates to decrease the resistance factor. A *fall in resistance* causes a reciprocal rise in the number of carrier individuals and in the amount of infectious material available. This *increase in the dosage* factor is followed by the epidemic outbreak. No significant enhancement in the virulence of the microbes is demonstrable. So consistent are these findings that their further operation was tested by similar studies of another animal disease.

Fowl cholera was chosen for study because it is regarded as a classical example of an epidemic disease. Its characteristics are well-recognized.

The disease occurs, according to Manninger (2), wherever poultry are raised in any great number. It is said to assume two forms: one, explosive, acute, and highly fatal, in communities hitherto unexposed; the other, a mild, endemic form attended by occasional, rather severe outbreaks. *P. avicida* is believed to exist both in a saprophytic form of low virulence and as a highly virulent parasite in the tissues of an occasional "carrier" fowl (2). The portal of entry is believed by field observers to be the gastrointestinal tract, although attempts to induce the disease experimentally by feeding have commonly failed (3). The lesions found in fowls dying of the acute form of disease are not extensive. They consist of sero-fibrinous exudate in the pericardial sac, subserous petechial hemorrhages in the pericardium and intestine, and congestion and sero-fibrinous exudate in the lungs. When the affected fowl survives longer, one finds fibrinous pneumonia and necrotic foci in the liver. In the chronic disease, caseous pneumonia, pulmonary abscesses, periarthritic, subcutaneous, and intramuscular abscesses are encountered. Moreover, localized lesions, such as edema of the wattle and some forms of nasal, sinus, and ocular roup have been considered manifestations of fowl cholera (4). *P. avicida* is known to possess definite characteristics common to the Pasteurella group. But the serological relationships of cultures from different sources and of Pasteurella organisms of other animals are poorly understood.

These facts summarize what is known of the epidemiology of fowl cholera. The present studies were designed to gain a more complete understanding of its mode of spread. For this undertaking, three general methods of investigation were employed: A) bacteriological and pathological tests; B) field observations, and C) studies of epidemics experimentally induced in poultry flocks. Each was applied to a definite phase of the general problem. The bacteriological and pathological tests were made to determine 1) the characteristics of strains of Pasteurella obtained from typical fresh cases of the disease, 2) the relation of these strains to the so-called "surface," "saprophytic" forms of the organism, 3) the portal of entry into the fowl, and 4) the varieties of host response to these microbes. A field study of the spontaneous disease in poultry flocks was undertaken to ascertain 1) the amount and character of the infection, 2) monthly fluctuations in its prevalence, 3) relation of *P. avicida* nasal carrier rate to spread of the disease, 4) the significance of different strains of *P. avicida*, and 5) differences in response of individual birds to the infection. An experimental study of fowl cholera epidemics is being made under controlled conditions whereby communities of healthy fowl are established and strains of *P. avicida* introduced, a technique permitting

direct determination and measurement of the factors concerned in the rise and fall of epidemic outbreaks.

The results of A) the special studies of *P. avicida* are presented in the following paper by T. P. Hughes (1 a); a preliminary report has already been issued (1 f). The findings are as follows: 209 strains of *P. avicida* from fresh cases of fowl cholera arising on widely separated poultry farms proved to be closely identical in cultural and serological behavior. On the basis of colony formation, however, three groupings were made: "fluorescent," "blue," and "intermediate" types. The "fluorescent" form, stable in suspension, with low, narrow zone of agglutination in acid buffers (pH 2.4–3.0), was found only in association with severe epidemics, and was relatively highly virulent and non-vegetative. The "blue" colony type, unstable in suspension, with wide zone of acid agglutination (pH 2.4–5.0), was found to be commonly associated with endemic cholera and was of relatively low virulence and high vegetative capacity. The "blue" colony type was obtained also *in vitro* from cultures of "fluorescent" type organisms grown under unfavorable conditions. The third colony type, designated "intermediate," proved somewhat stable in suspension, usually with a wide acid agglutination zone, and was "intermediate" in virulence and vegetative capacity. This variety was found in communities in which severe epidemics existed. Apparently the fluorescent colony form can be transformed into a blue colony type which may or may not be identical with the "blue" colony types found in communities where fowl cholera is endemic. Attempts to change "blue" colony types to "fluorescent" have been unsuccessful. The limit of variability of the "intermediate" colony type has not been determined. Under some conditions it resembles the "fluorescent" form; under others, the "blue," and yet attempts to effect a permanent transformation have not succeeded. The serological uniformity of *P. avicida* indicated by these studies may be apparent rather than real. Possibly, specific substances, undetected and not measurable, are present.

The results of special studies on portal of entry of *P. avicida* and host reaction to these organisms are presented in paper III by T. P. Hughes and I. W. Prichett (1 b), and in the preliminary report (1 f). They point out that fowl cholera is to be regarded as a respiratory infection which manifests itself in various ways by bringing about a)

a "carrier" state in the nasal passages of healthy individuals, b) an involvement of sinuses and nasal passages in others, as well as c) the typical manifestation of the acute septicemic process.

The results of B) field studies of the spontaneous disease in poultry flocks are contained in papers IV and V by I. W. Pritchett, F. R. Beaudette, and T. P. Hughes (1 c, d.) The findings differed according to whether the infection had been endemic in the flock for some time, or introduced relatively recently. The infection, when present in endemic form, varied with the season, increasing in amount and severity during the autumn months to a winter maximum, and then declining during the summer season to a minimum. Furthermore, its prevalence and tendency to spread varied with the number of nasal carriers: a decrease in carrier rate being accompanied by a decrease in disease mortality and in the occurrence of the local infections, roup, catarrh, and edema of the wattle. In these communities, the "blue" colony type of *P. avicida* was recovered. The virulence of strains from fatal septicemic cases, from local lesions of survivors, and from the nasal clefts of healthy carriers, was similar and relatively low. *P. avicida* infection in flocks of poultry with no history of previous cholera assumed the form of severe epidemics. The outbreaks arose during the winter months. The daily death rates were high; post-epidemic carriers among the survivors were uncommon. Following an epidemic, the infection tended to die out. The strains of *P. avicida* recovered were of both the "fluorescent" and "intermediate" colony types and of high virulence.

C) The observations on "experimental" epidemics of cholera are still in progress and are withheld for further consideration.

The studies so far carried out indicate that the epidemiology of fowl cholera rests upon essentially the same basis as that of rabbit pasteurellosis (1 e). In each instance, the severe epidemic form of infection is associated with a relatively virulent type of organism which survives with difficulty in the tissues of the host, whereas the endemic disease is associated with strains of relatively low virulence and high vegetative capacity. In fowl cholera, as in other animal diseases studied (1 e), the spread and severity of infection appear to be controlled by the resistance of the host and the dosage of the organisms.

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THE EPIDEMIOLOGY OF FOWL CHOLERA

II. Biological Properties of P. avicida

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Plate 6

(Received for publication, October 25, 1929)

At the outset of the present studies of fowl cholera, attention was directed to the etiological agent, *Pasteurella avicida*.[1] The early investigations of this organism are chiefly of historical significance; reports of its biological characteristics, its range of variation and adaptability, and indeed, of the means for its exact identification are incomplete and contradictory.

The unsatisfactory state of this information can be emphasized by a brief summary of the literature. Following the early investigations of Perroncito (1), Toussaint (2), Pasteur (3), and Kitt (4), *P. avicida*, together with similar bacteria from other animal hosts, were placed together and designated the "Pasteurella" or "hemorrhagic septicemia" group (5). But later investigators disagreed as to the properties of these organisms. Thus, *P. avicida*, according to Schirop (6) and others (7–12), ferments dextrose, saccharose, and mannite without the production of gas. Kaupp and Dearstyne (13), on the other hand, found dextrose alone to be acidified; Tanaka (14), maltose, in addition to the above; Higgins (15), acid production on lactose litmus agar. Indol formation in peptone water was observed by all the investigators mentioned, save Kaupp and Dearstyne (13) and Lignieres (16). Theobald Smith and others (17–18) report the organism to be highly virulent for fowl and rabbits; Turner (19) regards this as a diagnostic criterion. Wide discrepancies in results of serological studies are indicated by the reports of Bushnell (20), which state that less than 50 per cent of the strains examined were capable of acting as antigens, while Fitch and Nelson (21), on the other hand, divided their strains into definite groups according to their agglutination in specific sera.

Such conflicting reports made necessary a further study of *P. avicida*. To this end, 209 strains were collected from autopsies on

[1] Synonyms: *Bacillus avisepticus*, *Pasteurella avium*, *Coccobacillus avicida*, *Bacillus bipolaris aviseptica*, and *Pasteurella cholerae gallinarum*.

recent cases of undoubted fowl cholera. Thirty-one of these strains came from a commercial poultry farm on Long Island (Paper IV), 172 from six scattered farms in New Jersey, 4 from poultry in Northern New York, and 2 from Kansas. One further strain, "Pa," obtained from Dr. Theobald Smith, came originally from a farm in Pennsylvania. These 210 strains were submitted to routine and special examinations, the results of which are contained in the following paragraphs.

General Characteristics

Morphology. All strains appeared as small, rounded, bipolar bacilli, decolorizing in Gram stain and were non-motile. The bacilli seemed relatively large in blood films from infected fowls, and in films stained by Wright's method (Fig. 1). Involution forms occurred in old cultures, small, faintly colored, coccoid forms predominating. Attempts to demonstrate capsules with special stains were not successful, but on numerous occasions the highly virulent, so-called "fluorescent" colony forms showed a clear peripheral zone resembling that of an unstained capsule.

Growth in Fluid Media. The bacilli grew well in infusion broth plus a trace of hemolyzed rabbit or chicken blood. Four hours after loop inoculation of the fluid, turbidity was perceptible; after fifteen hours, it was maximal. Pellicle formation appeared occasionally in 3–4 day cultures, but "granulation" and sedimentation did not occur. Asparagin synthetic media did not support growth; Dunham's peptone water gave feeble and retarded growth. Growth rate was enhanced, however, when unheated fresh chicken tissue was added to infusion broth.

Carbohydrate Fermentation and Indol Production. The carbohydrate media was made up of Dunham's peptone solution plus 0.5 per cent sugar and 1.0 per cent Andrade's indicator. This was tubed in 5 cc. quantities, together with a small, inverted tube to detect gas production. All strains acidified dextrose, saccharose, and mannite without the formation of gas. The acidity was slight and tended to decrease as artificial cultivation was prolonged. One exception to this behavior was noted in the case of twenty strains from a single flock which showed, in addition to the above, acid in xylose. All strains

gave a positive indol test when grown 2+ days in Dunham's peptone and examined by Ehrlich's method.

Colony Morphology. The cultures when grown on infusion agar plus a trace of hemolyzed blood showed three distinct forms of colony. Colony morphology was found later to be associated with other definite properties and came to be used as a means of classification. Hence, considerable attention was devoted to the behavior of these strains on solid media.

The first colony type was designated "fluorescent." It is relatively large and when seen by transmitted light, is opaque and fluorescent. By reflected light, it is greyish-white and moist (Fig. 2). Three strains from the Long Island farm, those from northern New York, the Kansas, and "Pa" cultures, all "epidemic" strains, formed fluorescent colonies.

The second colony type was called "blue." It is much smaller than the fluorescent form, relatively transparent, grey-blue, and without fluorescence (Fig. 2). All strains from the New Jersey flocks in which fowl cholera was endemic were of the "blue" colony type. This form occured also when the fluorescent type strains were grown for extended periods in artificial media or with specific antisera.

The third form of colony has been termed "intermediate," because of its partial resemblance to both the "fluorescent" and "blue" types. Under optimum conditions it may be described as similar to the "fluorescent" colony, but less opaque and less fluorescent. Colony appearance of the "intermediate" type is variable, however. When fifteen hours old, it seems typically "fluorescent;" at forty-eight hours, quite "blue." When colonies of this type are crowded, those at the periphery appear "fluorescent" at twenty-four hours, and the central ones "blue;" later, all become "blue." Frequently "ring" colonies are seen, composed of a "blue" core and "fluorescent" ring. The "intermediate" colony type strains were obtained from a single farm on Long Island during epidemic and post-epidemic periods.

The permanency of these colony types was tested on media, in sera, and in the animal body. When passed on solid media, "blue" colony forms remained "blue," "intermediates" varied as described in the preceding paragraph, but did not actually change to either of the other two types. The "fluorescent" type, on the other hand, did not

alter on blood agar, but, on infusion agar after eight passages, showed a mixture of "fluorescent" and "blue" colony forms. In fluid media the variations were similar, except that "fluorescent" types changed to "blue" more rapidly. Passage in antisera did not change the colony form of the "blue" nor "intermediate" type, but the "fluorescent" forms were replaced by "blue" colonies. Direct passage in fowls by intrathoracic injection failed to change the colony types. At present, therefore, it seems that "blue" colony types are permanent, and that "intermediate" forms as well, although varying in appearance, do not change their type. "Fluorescent" colony strains, on the other hand, growing under unfavorable circumstances, change to the "blue" colony form.

Acid Agglutination Zone. The behavior in acid buffer solutions of seventy strains was tested according to the method of Northrop and de Kruif (22). On centrifuging it was found that the "blue" colony type strains sedimented easily, whereas suspensions of the "fluorescent" colony strains were very stable. This difference was in accordance with the results of the acid agglutination tests (Table I) which showed a characteristic zone for each type. The "fluorescent" colony strains fluocculated at pH 2.4 or not at all. The "blue" colony strains, on the contrary, showed either a wide zone, pH 2.4–5.4, or a zone well toward the alkaline side. Flocculation of the "intermediate" colony forms was limited to a more narrow region toward the acid range of the scale, pH 2.4–4.0.

Serum Agglutination. The antigenic relationships of the 210 strains were studied by direct and reciprocal agglutination and absorption tests.

Antisera were prepared by injecting rabbits intravenously with washed saline suspensions of organisms taken from agar plates and preserved with 0.2 per cent phenol. The injections were given on five successive days, with doses increasing regularly from 0.1 to 0.5 cc. Two additional injections of 0.5 cc. were administered at five day intervals. After a final rest period of ten days, the animals were bled.

The first test was made with a "Pa" strain antiserum. Antigen-antibody mixtures were read after a three hour period in a water-bath at 56°. All but ten strains were agglutinated. These non-agglutinating cultures were either "fluorescent" or "intermediate" colony forms with narrow acid agglutination zones.

Further tests were made with the "fluorescent" and "intermediate" colony strains to determine if possible whether they were antigenically unrelated to the other cultures or combined with specific antibody and unable to flocculate. In the first place, it was found that "blue" colony variants from the "fluorescent" forms agglutinated to titre in the "Pa" serum. Again, antisera prepared from two "fluorescent" and two "intermediate" colony forms were ineffective toward their

TABLE I

Acid Agglutination of P. avicida

Colony type	Source	Range of pH	Number of strains
Fluorescent	Autopsy	No agglutination	2
"	"	2.4 only	1
Blue variant	Fluorescent	2.4–5.4	2
" "	"	2.4–4.6	1
Blue	Autopsy	2.4–5.4	18
"	"	2.4–5.0	1
"	"	3.0–5.0	22
"	"	3.0–5.4	5
Intermediate	"	2.4 only	2
"	"	2.4–3.0	1
"	"	2.4–3.2	2
"	"	2.4–3.5	1
"	"	2.4–4.2	1
"	"	3.0–4.0	3
"	"	3.0–4.2	7
"	"	3.2–3.5	1

homologous antigens, but brought about strong agglutination of the "Pa" strain. Finally, reciprocal absorption tests were made according to the method of Krumwiede (23) to determine whether the non-agglutinating strains could absorb agglutinins from heterologous sera and, conversely, whether the heterologous strains could absorb agglutinins from the sera of the non-agglutinating strains. The results of three such tests are given in Table II. Strains 631 and 638 are representative of the "fluorescent," non-agglutinating form; Strain 728 is an example of the "blue" agglutinating form; Strain

TABLE II

Reciprocal Absorption Tests with P. avicida

Serum	Antigen	Result
	"Pa" and 728	
"Pa"—unabsorbed	"Pa"	Agglutination 1:320
" "	728	" 1:320
" —absorbed with "Pa"	"Pa"	No agglutination.
" " " "	728	" "
" " " 728	"Pa"	" "
" " " "	728	" "
728 —unabsorbed	"Pa"	Agglutination 1:320
" "	728	" 1:320
" —absorbed with "Pa"	"Pa"	No agglutination.
" " " "	728	" "
" " " 728	"Pa"	" "
" " " "	728	" "
	"Pa" and 631	
"Pa"—unabsorbed	"Pa"	Agglutination 1:320
" "	631	No agglutination.
" —absorbed with "Pa"	"Pa"	" "
" " " "	631	" "
" " " 631	"Pa"	" "
" " " "	631	" "
631 —unabsorbed	"Pa"	Agglutination 1:1280
" "	631	No agglutination.
" —absorbed with "Pa"	"Pa"	" "
" " " "	631	" "
" " " 631	"Pa"	" "
" " " "	631	" "
	"Pa" and 638	
"Pa"—unabsorbed	"Pa"	Agglutination 1:320
" "	638	No agglutination.
" —absorbed with "Pa"	"Pa"	" "
" " " "	638	" "
" " " 638	"Pa"	" "
" " " "	638	" "
638 —unabsorbed	"Pa"	Agglutination 1:600
" "	638	No agglutination.
" —absorbed with "Pa"	"Pa"	" "
" " " "	638	" "
" " " 638	"Pa"	" "
" " " "	638	" "

"Pa" an "intermediate" agglutinating form. Strains 631 and 638 did not agglutinate in homologous or heterologous sera; however, they did absorb agglutinins from homologous and heterologous sera. Furthermore, antibodies in sera from these non-agglutinating strains were absorbed by the homologous and heterologous strains. The test with the "blue" form 728 is included as a control, showing the complete absorption of agglutinating strains.

The antigenic relationship of chicken and rabbit strains of Pasteurellae was tested by titrating a) rabbit cultures, Rivers D and DC 30 (29) in chicken strain antisera 638 and "Pa," and b) fifteen fowl cholera strains, representing "blue," "fluorescent," and "intermediate" colony forms in rabbit strain, Rivers D, antiserum. No agglutination occurred, indicating that the fowl cholera strains differ antigenically from the rabbit strains tested.

Complement Fixation. To test further the antigenic relationship of strains of *P. avicida*, the complement fixation reaction was employed. Thirty strains were examined for their complement fixing power with "Pa" serum and a lesser number with sera from non-agglutinating strains previously described.

In general, the method described by Bull (24) was followed. The organisms to be used as antigens were well washed and suspended in salt solution. Their anti-complimentary titre was determined by finding the amount of suspension that would destroy the complement in 0.2 cc. pooled guinea pig serum diluted 1:10. The mixture of complement and bacteria was incubated at 37° for one hour, then tested with anti-sheep cell hemolytic system. One-third of the anti-complimentary titre was used as the antigenic dose in the final test. This amount of suspension was mixed with 0.5 cc. of the "Pa" serum, used in dilutions from 1:16 to 1:1200. Two units of complement were added, the tubes brought to a uniform volume of 1.5 cc., allowed to incubate at 37° for one hour, then left overnight in the icebox. The hemolytic system was added and incubated for one hour at 37°. Tubes in which hemolysis was entirely inhibited were considered positive.

The results of these complement fixation tests are given in Table III and compared with corresponding agglutination titres. The titre of various strains with other sera is presented in Table IV. The inagglutinable "fluorescent" colony strains proved fairly reactive in their ability to fix complement, and the "Pa" antigen fixed complement in various sera to the same extent as the homologous strains.

TABLE III

Results of Complement Fixation and Agglutination Tests

	Number of strains	Maximum effective dilution of "Pa" serum	
		Agglutination	Complement fixation
Fluorescent form	1	0	25
	1	0	256
	1	0	40
Blue form	1	1280	160
	1	1280	320
	2	2500	320
	2	2500	160
	2	2500	512
	1	2500	640
Intermediate form	2	0	256
	1	40	20
	1	50	40
	1	80	25
	2	80	50
	1	80	75
	1	160	40
	4	160	50
	1	160	160
	1	320	50
	3	640	640

TABLE IV

Complement Fixation Tests with Organisms of Different Colony Forms

Antigens	Maximum serum dilution showing complete fixation				
	Sera				
	"Pa"	770	773	629	638
"Pa"	50	75	200	300	900
770 ("Blue")	75	25	100	50	200
773 ("Blue")	50	100	200	100	200
629 ("Fluorescent")	40	50	100	100	600
638 ("Fluorescent")	20	25	75	100	600

These results are regarded as further evidence of the antigenic similarity of strains of *P. avicida*.

Toxin Formation. Numerous unsuccessful attempts were made to demonstrate the presence of a toxin resembling that described by Pasteur (25).

Virulent bacilli were grown in infusion broth, with and without the addition of hemolyzed blood and fresh, unheated chicken tissue. The cultures were incubated aerobically and anaerobically for periods of eight hours to one week. The filtrates were tested by intravenous, intramuscular, and subcutaneous injections into chickens. Some filtrates were concentrated to one-tenth volume by distillation *in vacuo* and then tested. A further series of inoculations was made with bacilli dissolved in dilute alkali. In no instance did the inoculated animals die, nor symptoms develop resembling those of fowl cholera.

Virulence. From an epidemiological point of view, no property of bacteria is more important than that of virulence or pathogenicity. Hence in these studies an effort has been made to determine with care the disease-producing power of *P. avicida.*

The test animals were chickens from a single flock of healthy breeding hens, shipped to this laboratory one day after hatching and raised under uniform conditions. Twenty birds, four weeks old, weighing 100–150 gms., were used for each test. The bacterial cultures for testing were grown on hemolyzed blood agar for 15–18 hours, washed off, and suspended in infusion broth. The density of the suspension was standardized with the Gates' turbidometer (26) and brought to the concentration of an 18 hour culture. The dose employed was 0.2 cc., controlled by counting, and representing about 20,000,000 bacilli. The inoculum was deposited from a syringe fitted with a blunt pointed needle, on the intact nasal mucosa. The reasons for using the intranasal portal of entry are discussed in the following paper. After inoculation, each bird was kept in a separate cage. The tests extended over a ten day period, although deaths were infrequent after the fourth day.

To control the effect of variations in susceptibility of the chickens, one strain of "fluorescent" colony type organisms was reinjected into a control group of twenty birds with each test.

The results of these titrations are given in Table V, where "virulence" is expressed as percentage of birds dying of fowl cholera. Other determinations, if made on the identical culture, are included. The data indicate a distinct correlation between virulence and the attributes associated with colony form. Thus, the "blue" colony types proved relatively avirulent, while the "fluorescent" forms were highly pathogenic. Single tests on the "intermediate" colony strains gave

results varying from 0 to 60 per cent mortality. For this irregularity, three explanations are possible,—technical difficulties, variations in the resistance of the birds, or actual differences in the virulence of the "intermediate" strains. Data to be presented in the succeeding

TABLE V

Virulence and Other Properties of Pasteurella avicida of Different Colony Forms

Colony form	Source	Strain	Acid agglutination (pH range)	Serum agglutination (Maximum dilution)	Virulence (Per cent mortality)
Fluorescent	Autopsy	629	2.4 only	0	10
		631	none	0	60 (av. 4)
		638	none	0	30 (av. 5)
Blue variant	Fluorescent	629 B	2.4–5.4	1280	0
		631 B	2.4–4.6	2500	0
		638 B	2.4–5.4	1280	0
Intermediate	Autopsy	729	3.0–4.2	2500	0
		730	3.0–4.2	2500	0
		731	3.0–4.2	1280	0
		733	2.4–3.5	320	0
		726	2.5–4.1	100	10
		798	2.5 only	0	0
		796	2.5–4.7	640	0
		635	3.0–5.4	1250	20
		641	2.4–5.0	1280	20
		642	2.4–5.4	640	60
		644	2.4–5.0	2500	20
		648	3.0–5.0	2500	40
Blue	Autopsy	651	3.0–5.0	5000	0
		647	2.4–5.0	640	0
		702	2.4–5.0	640	0
		773	2.4–5.4	5000	10
		779	2.5–5.2	640	0
		793	2.5–5.2	1250	0
		781	2.5–5.2	1250	20

papers render the latter possibility unlikely and indicate that strains of a given colony type are in general of similar virulence. The relative pathogenicity of "fluorescent," "intermediate," and "blue" colony types may be expressed for convenience as 50 per cent, 25 per cent, and 0 per cent respectively.

Attempts to raise virulence by growth in media, sera, and passage through fowls were not successful. "Blue" colony forms could not be changed to "intermediate" or "fluorescent," nor could an increase in virulence of any strain be demonstrated. Tests by means of host passage were made by intranasal instillations with and without the use of brilliant green (Bull, 27). One test at a season when titration mortality was low was carried on for seven passages (Table VI). The strain passed directly in blood from bird to bird showed no increase in virulence.

TABLE VI

Attempt to Increase Virulence by Chicken Passage

Passage	Preliminary treatment of chicken	Survival time in days	Form of organism recovered	Virulence
I	Brilliant green intranasally	2	"Fluorescent"	
II	" "	12	"	
III	None	6	"	
IV	"	6	"	
V	"	3	"	
VI	"	3	"	5 chicks inoculated, 1 died.
VII	"			5 chicks inoculated, none died.

DISCUSSION

This study of 210 freshly isolated strains from known cases of fowl cholera has proved useful in defining and classifying the inciting microbe of fowl cholera, *P. avicida*. This definition and classification were made on the basis of general uniformity in morphology and growth characteristics in fluid media and like reactions in carbohydrates and indol production. By the aid of serological methods, a fundamental antigenic similarity of all strains of *P. avicida* has been determined. The extent of this specificity is, however, unknown. It may indicate antigenic identity of all strains or the presence of a common antigenic fraction among otherwise distinct serologic groups similar to the "protein" fraction of pneumococci (28). At present, however, further analysis is hindered by the apparent inagglutinability of certain otherwise typical strains.

However, a temporary classification of *P. avicida* is suggested on the basis of colony morphology. One type, "fluorescent," is associated with epidemics of fowl cholera, is highly virulent, stable in suspension, agglutinable only in very acid buffers, pH 2.4, and not at all in antisera. It resembles the "D" form of *P. lepiseptica* (29). The second type, "blue," is found in flocks where fowl cholera is endemic, is of low virulence, unstable in suspension, agglutinates in acid buffers over a wide zone, pH 2.4 – 5.6+, and in all *P. avicida* antisera. This type corresponds to the "G" form of *P. lepiseptica* (29). The third type, "intermediate," is associated with the more severe fowl cholera, and is "intermediate" in its behavior. This form bears some resemblance to the mucoid types of *P. lepiseptica* (29). These groupings have remained consistent throughout the present studies.

Hence there is available a definite criterion for the diagnosis of *P. avicida*, together with a sub-grouping of considerable epidemiological importance.

SUMMARY

1. A bacteriological study has been made of 210 fresh strains of Pasteurella obtained from typical cases of fowl cholera on seven widely separated poultry farms.

2. The strains have proved identical in consisting of small, pleomorphic, bipolar staining, Gram-negative, non-motile bacilli. They grew rapidly in infusion broth plus a trace of hemolgobin. They formed acid but no gas in media containing dextrose, saccharose, and mannite; indol was produced.

3. The strains fall into three distinct groups, according to their colony formation on hemoglobin agar. The "fluorescent" colony was large, whitish, opaque, exhibiting, under suitable conditions marked fluorescence. The "blue" colony was smaller, clear slate-blue, and non-fluorescent. The "intermediate" colony was moderately fluorescent at 15–18 hours growth, and "blue" thereafter. It was "blue" at all times when crowded and occasionally of "ring" form. "Fluorescent" colony cultures developed "blue" colony forms under certain conditions; otherwise all forms were stable.

4. Strains from "fluorescent" colonies were resistant to precipitation by acids, to sedimentation by centrifuging, and although they com-

bined with specific antiserum, did not agglutinate. They were relatively highly virulent and occurred in flocks where fowl cholera was epidemic.

5. Strains from "blue" colonies were precipitated by acids over a wide range of concentration and agglutinated strongly in antisera. They were relatively of low virulence and occurred in flocks where fowl cholera was endemic.

Strains from "intermediate" colonies varied in behavior betweeen "fluorescent" and "blue" strains. They came from a flock where fowl cholera was epidemic.

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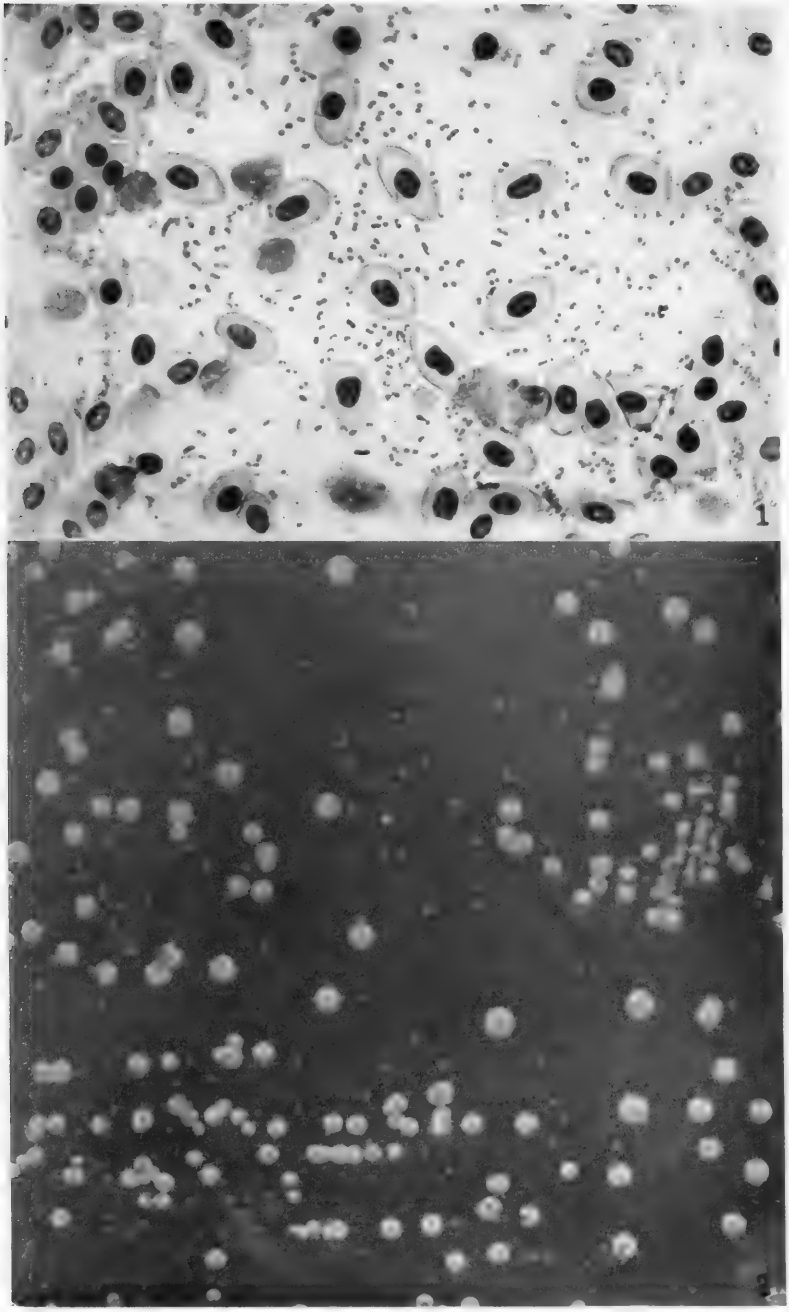
EXPLANATION OF PLATE 6

FIG. 1. *P. avicida* in blood of experimentally infected fowl. Antemortem specimen.

FIG. 2. "Fluorescent" and "blue" colony forms of *P. avicida*.



(Hughes: Epidemiology of fowl cholera. II)

THE EPIDEMIOLOGY OF FOWL CHOLERA

III. Portal of Entry of P. avicida; Reaction of the Host

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Plate 7

(Received for publication, October 26, 1929)

The part of these studies dealing with the identification and characteristics of *P. avicida* has been reported in previous papers. It is proposed now to describe a number or tests made to determine: a) the normal portal of entry of these organisms into the chicken host, and b) the forms of infection so established.

Various portals of entry for the spontaneous infection have been suggested, but the gastrointestinal route has been considered as the most likely (Kolle u. Wassermann, III Aufl.). However, experimental infection by feeding methods is known to be difficult. Hertel (1) and Muller (2), for example, reported that when the inoculum is instilled directly into the esophagus of fowls and pigeons, in such a way as to avoid contact with the nasal and pharyngeal mucosae, or given in the relatively dry form of a pellet, infection does not take place. Nevertheless, the opinion of field workers that the disease is gastrointestinal in nature is generally accepted. The clinical course of acute fowl cholera and the pathological findings at autopsy are fully described in the literature (Kolle u. Wassermann, III Aufl.). But references to chronic cases are exceptional and the existence of localized infections is recognized only in occasional reports (3–9).

In contrast to this incomplete knowledge of *P. avicida* infection are the facts relative to infection by a similar organism, *P. lepiseptica* (10). These bacilli are known to enter the rabbit host by way of the upper respiratory tract and to induce various forms of localized and general disease.

Accordingly, tests were planned to determine whether the natural portal of entry of *P. avicida* into fowls was by way of the mouth or nasal passages, and whether in fowl cholera as in rabbit snuffles-pneumonia, infection appeared in various generalized and local forms.

Normal Portal of Entry

Preliminary tests showed that cultures of *P. avicida* administered *per os* to chicks and adult fowl did not infect, whereas when dropped into the nasal cleft, they brought about the typical disease (Table I–IV). The details of these tests are as follows:

Two fluorescent strains, "Pa" and "Kansas," described in the previous paper (11), were employed. The dose 0.2 cc., and the method for its control have been discussed (11). The birds were either five

TABLE I

Comparative per Os and Intranasal Injection of P. avicida into Fowl

Test	Number of birds injected	Inoculum	Number dead	Per cent dead
I	15—adults	0.2 cc. "Pa" *per os*	0	0
	" "	0.2 cc. "Pa" intranasal	6	40
II	25—adults	0.2 cc. "Pa" *per os*	0	0
	" "	0.2 cc. "Pa" intranasal	7	28
III	12—pullets	0.5 cc. "Pa" *per os*	0	0
	" "	1 drop "Pa" intranasal	4	33
IV	20—adults	0.2 cc. "Kan" *per os*	0	0
	30— "	0.2 cc. "Kan" intranasal	15	50
V	25—chicks	0.2 cc. "Kan" *per os*	0	0
	50— "	0.2 cc. "Kan" intranasal	22	44
VI	20— "	0.2 cc. "Kan" *per os*	0	0
	" "	0.2 cc. "Kan" intranasal	11	55

weeks chicks, similar to those already described, or adult pullets from the same source (11). It is important to mention again that these fowls had had no previous exposure to *P. avicida.* Intranasal inoculations were made by holding the bird head downward and instilling the desired amount of inoculum from a syringe through a blunt needle over the surface of the mucosa on the roof of the nasal cleft. The *per os* instillations were made by placing the inoculum in a small gelatin capsule and introducing it at once into the esophagus of the chicken with a pair of forceps. After injection each bird was placed

in an individual cage and observed for ten days. Autopsies were performed on all fatal cases and cultures taken for identification.

Tests V and VI (Table I) were subjected to the following additional control, to demonstrate that cultures introduced in capsules were liberated and had retained their virulence. A number of additional birds were given the capsules, and every fifteen minutes thereafter one bird was killed, autopsied, and cultured. The specific strains of *P. avicida* were recovered from crop and upper intestinal tract of the autopsied bird, and then reinoculated intranasally in the usual manner into another series of normal chicks. These reinoculated strains were found to possess the same virulence as the control cultures. To prove that birds inoculated *per os* were as susceptible as those inoculated intranasally, they were reinoculated after ten days by the intranasal route. The mortality following the introduction of organisms by the nasal route was similar to that of the control intranasal series. It was concluded from the tests that *P. avicida* does not infect by way of the gastrointestinal tract. On the other hand, the chickens which succumbed to the intranasal instillations presented the usual clinical and pathological lesions typical of fowl cholera. This was regarded as evidence that the natural portal of entry of *P. avicida* is by way of the upper respiratory tract.

Reaction of the Host

To gain more information on the mode and route of infection of *P. avicida*, and especially to study the varieties of host response, series of birds were given intranasal doses of the organisms.

The first tests were made to determine the extent of variation in per cent mortality of groups of similar birds given similar doses of the same strain of *P. avicida*.

"Fluorescent" colony strains, "Pa," "638," and "Kansas," and "intermediate" colony strains "629" and "631" were prepared as usual (11) and instilled intranasally in standard doses, 0.2 cc., 20,000,000 bacilli. The birds employed were 4–5 weeks old, 100–150 gms. chicks, raised under uniform conditions and free of previous exposure to *P. avicida*. At least twenty were used for each titration. The results of these tests are given in Table II.

Nine titrations of the "Pa" strain showed an average mortality of 31.9 ± 2.6 per cent. This average figure does not include the two

low values of May 16 and 23. Three titrations of "638," 40 ± 4 per cent; nine of "Kansas," 50.5 ± 5 per cent; three of "629," 16 ± 4.5 per cent, and four of "631," 42.5 ± 8.7 per cent. The small variations in per cent mortality between tests indicate that the technique was adequate, and that under these conditions the virulence of

TABLE II

Comparison of Results of Repeated Virulence Tests on Same Strains
(5 weeks chicks; dose 0.2 cc. culture, intranasally)

Date	Strain	Number injected	Per cent mortality	
March 29, '27	"Pa"	63	35	
April 4		21	29	
11		21	33	
18		27	29	Average* 31.9
May 3		56	31	Deviation =
5		50	30	2.6
9		66	29	
16		22	14	
23		30	13	
Oct. 27, '28		10	40	
Dec. 8		13	31	
Feb. 6	629	10	10	Average 16.6
March 10		10	20	
25		10	20	
March 8	631	10	40	Average 42.5
21		20	35	
April 11		10	60	
May 2		20	35	
Jan. 29	"Kansas"	10	60	
Feb. 4		50	44	
6		30	50	
8		20	65	Average 50.5
14		35	46	Deviation =
April 24		10	40	5.0
27		10	50	
28		10	50	
May 7		20	50	

* This average does not include the low determinations of May 16 and 23, 1927.

each strain was constant and the average resistance of the groups of chicks the same. Tests with adult birds gave similar results.

The possible effect of season is shown in the mortality figures of the "Pa" series. The drop in death rate in May from a relatively constant level of 30 ± to 15 per cent, and the subsequent rise to this level again in October is a phenomenon similar to those occurring in mouse typhoid, mouse pneumonia, and rabbit pneumonia infections (12).

The effect of dosage on mortality is shown in Table III. For this test, each of six groups of ten birds were given, in uniform volume, a definite dose of *P. avicida*, "Kansas," ranging from ten times the standard amount of 20,000,000 bacilli to 1/10,000 of this amount. The resulting mortality indicates that groups of chicks react similarly

TABLE III

Effect of Varying Dosage on Mortality

(0.2 cc. "Kansas" strain, intranasally, into groups of 10 chicks)

Dose	Per cent mortality
Standard (equal to 18 hr. broth culture)	40
10 times standard	80
Standard diluted 1:10	50
" " 1:100	40
" " 1:1000	40
" " 1:10,000	40

to doses between 20,000,000 and 20,000 bacilli, but are more susceptible to greater amounts and less susceptible to smaller numbers. Two additional tests of this sort gave similar results.

The response of young birds to the intranasal instillations of *P. avicida* took two forms,—either they died abruptly of typical fowl cholera, or remained quite healthy. None showed chronic or local forms of infection. Nevertheless, evidence was obtained indicating that *P. avicida* infection in nature and under proper experimental conditions takes the form not only of acute septicemic fowl cholera, but also of chronic and localized disturbances.

Indirect evidence of local and of chronic *P. avicida* infection came in part through the kindness of Dr. Arnold Branch and William Steenken, Jr., and is presented with their permission at this time.

A small flock of nineteen fowl maintained at Trudeau for studies on avian tuberculosis became infected "spontaneously" with *P. avicida*. Within a few

TABLE IV

P. avicida Infection in a Flock of Chickens at Trudeau, N. Y.

No.	Course of disease	Autopsy findings		Occurrence of *P. avicida*				
		External	Viscera	Blood	Wattle	Roup	Eye	Pericardium
A 854	Acute		Pericarditis Liver degeneration	+				+
C 33	Acute		Liver degeneration	+				
A 861	Acute		Liver degeneration	+				
A 853	Acute	Caseous wattle	Pericarditis Liver degeneration	+				+
C 34	Acute	Wattle disease	Pericarditis	+	+			+
A 858	Acute	" "	Pericarditis Pneumonia	+	+			+
A 852	Acute	Wattle disease; roup	Focal hemorrhage in lung	+				
C 31	Acute		Pericarditis	+				
A 863	Acute	Wattle disease	Pericarditis Perihepatitis	+				+
C 32	Acute	" "	Caseous lung focus	+	+			
B 219	Chronic	Rhinitis; roup		+		+		
B 218	Chronic	Roup		0		+		
E 31	Chronic	"		+		+		
B 222	Chronic	Roup; intraorbital abscess		0		+	+	
E 32	Chronic	Roup		0				
B 217	Chronic	Roup; rhinitis	Caseous nodules in liver	0		+		
B 220	Chronic	Roup		0		+		
B 223	Chronic	"		0				
B 221	Chronic	"		0				

days 50 per cent died of typical fowl cholera and were autopsied. Positive blood cultures were obtained in all cases. Six of the number showed localized wattle

disease and one roup. Cultures taken from three of the infected wattles yielded the epidemic strain of *P. avicida*. Nine of the birds survived but not without sickness and loss of weight. After a few weeks they were killed and autopsied. Two had positive blood cultures. All showed severe upper respiratory lesions, manifested clinically as mucopurulent discharge, roup, rhinitis, and orbital abscess. From the lesions of six of these cases, *P. avicida* was recovered. One bird showed a chronic pulmonary lesion; another a similar focus in the liver. The strains sent to the Rockefeller Institute laboratory appeared identical; no distinction was possible between cultures from local lesions of chronic cases and blood cultures of acute "cholera" cases.

An additional study of "spontaneous" local lesions was made on birds from poultry farms in New Jersey. Lesions from twenty-seven

TABLE V

Bacteriological Examination of Local Infections Occurring in Chickens (a) "Spontaneously" and (b) Following Intranasal Inoculation with P. avicida

Lesion	From "spontaneous" lesions		From lesions occurring in experimentally inoculated birds	
	Positive for *P. avicida*	Negative for *P. avicida*	Positive for *P. avicida*	Negative for *P. avicida*
Ocular roup	7	3	7	0
Mixed ocular and sinus roup	0	1	0	0
Sinus roup	0	5	4	1
Edema of wattle	5	3	1	1
Otitis media	2	0	8	0
Submandibular abscess	1	0	0	0

birds on a number of different farms were cultured; fifteen yielded *P. avicida* of the "blue" colony type (Table V) (Figs. 1 to 3). Of these, nine were tested for virulence and, as usual, failed to kill.

Direct evidence of chronic and localized *P. avicida* infection was obtained by giving cultures intranasally to tested non-carrier, healthy fowl. In all, twenty-two local lesions occurred, classified as roup, edema of wattle, and abscess. Twenty of these proved positive (Table V). It is concluded, therefore, that roup, edema of the wattle, and sinusitis in fowl are frequently local forms of *P. avicida* infection.

One other form of *P. avicida* infection was studied,—the carrier

state. That a certain number of birds in infected flocks carry the organisms in the nasal cleft will be brought out in the following paper; at this time it will suffice to present the results of tests on birds given the organisms experimentally by the nasal route. Birds were tested before inoculation and at intervals thereafter (Table VI). The results show that the carrier state is a definite, though clinically

TABLE VI

Occurrence of Carriers among Injected Birds

Date	Strain	Number injected	Per cent mortality	Per cent carrier
April 4	642	10	30	0
February 7	642	10	60	0
	648	10	40	0
March 8	631	10	40	10
	635	10	30	10
	638	10	50	10
	651	10	50	0
	661	10	30	0
	667	10	40	0
	672	10	40	0
April 11	631	10	60	0
April 18	661	10	30	0
April 5	660	10	0	20
	690	10	0	30
	735	10	0	10
	793	10	0	10
	629 B	10	0	20
March 22	745	10	0	20
	749	10	0	10
	773	10	0	20
March 29	770	10	10	30
	779	10	0	20
	785	10	10	10

concealed form of *P. avicida* infection, and that it occurs most frequently with the "blue" type, so-called endemic forms, and rarely with the "fluorescent" colony "epidemic" types.

DISCUSSION

The data contained in this report indicate that fowl cholera is not primarily a gastrointestinal disease, but that, like rabbit pasteurellosis

(12), it is upper respiratory in origin. These observations show further that the etiological agent, *P. avicida*, gives rise not only to the well-known septicemic cholera, but also to various types of chronic pneumonia and liver abscesses, and local disturbances of the upper respiratory tract, such as roup, sinusitis, and inflammation of the wattle. Still another form of infection, clinically unrecognizable, is the "healthy" nasal-carrier state. Finally, it appears that of any group of healthy, selected chickens given a similar dose intranasally, some will resist infection entirely.

The fact that chickens under controlled conditions react differently to the same intranasal dose of *P. avicida* indicates that they differ in their ability to resist the infection and that, as in the case of rabbits exposed to *P. lepiseptica* (13), the most resistant individuals are not infected; those less so become carriers, others more susceptible contract local upper respiratory lesions, while the most susceptible die of the acute generalized infection.

Another observation of importance is that a given strain of *P. avicida* given intranasally to a number of groups of controlled and selected birds gives rise, in general, to a similar amount of mortality. From this the conclusions are drawn that the *technique* of titration is adequate for measuring virulence of organisms, and that under these conditions the *virulence* of a given strain and the *average resistance* of the chickens are constant. The summer decrease in mortality is at present considered due to an increase in the resistance of the birds.

The standard dose, estimated to be approximate to that available to flocks at epidemic times, may be increased ten times, or decreased 1000 times without apparent effect on mortality. Outside of those limits, however, dosage influences mortality. These observations are in accord with similar tests with mouse Friedländer (14) and typhoid (15) bacilli.

SUMMARY

1. In the experiments here presented, *P. avicida* proved incapable of inciting fowl cholera when introduced directly into the alimentary tract. On the other hand, when administered into the upper respiratory passages, it induced typical disease.

2. When *P. avicida* was introduced into the nasal passages of con-

trolled, selected chickens, some died of typical septicemic cholera, a few developed chronic pneumonias and other conditions and succumbed, a few developed localized upper respiratory inflammations, such as rhinitis, roup, and wattle involvement, while yet a few others became "healthy" nasal carriers. Usually, however, more than 50 per cent resisted infection. Repeated titrations of this sort gave, in general, uniform results, save that in spring and summer the per cent mortality decreased.

3. *P. avicida* was recovered from a number of cases of "spontaneous" roup, rhinitis, and wattle disease.

4. Groups of chickens reacted similarly to doses of virulent *P. avicida* varying from 20,000,000 to 20,000. Outside these limits, dosage exercised a marked influence on mortality.

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EXPLANATION OF PLATE 7

Spontaneously occurring lesions yielding *P. avicida* on culture.

FIG. 1. Wattle disease.

FIG. 2. Ocular roup. The lesions on the comb are due to fowl pox.

FIG. 3. Sinus roup.

(Hughes and Pritchett: Epidemiology of fowl cholera. III)

THE EPIDEMIOLOGY OF FOWL CHOLERA

IV. Field Observations of the "Spontaneous" Disease

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(Received for publication, October 26, 1929)

The results of special studies on the bacteriology of fowl cholera, its mode of infection, and reaction of the host have been dealt with in the preceding papers (1 a, b). It is proposed now to present the data collected from field studies as the disease occurs spontaneously in poultry flocks.

Methods

A series of expeditions was made to the various poultry farms chosen for study. At each trip, a census was taken, the amount of clinical infection noted, and cultures made from the nasal passages of all or part of the flock. These cultures were then brought to the laboratory at New Brunswick or New York for study. In addition to this, birds dying within the period of observation were sent to New Brunswick if possible, and there autopsied and cultured.

To test for carriers, sterile cotton swabs of appropriate size were passed over the nasal mucosa and streaked on hemolyzed blood agar contained in 150 cm. Petri dishes. After 18–24 hours' incubation, the number of colonies of *P. avicida* on the plates was estimated and suspicious colonies transferred to appropriate media for identification and colony type determination. Cultures showing characteristic colony appearance and morphology, acid production in dextrose, saccharose, and mannite, and a positive indol test were considered *P. avicida*.

Individual birds in the flock were identified by numbered leg bands.

A Study of Epidemic P. avicida Infection

This investigation was initiated by the bacteriological examination of three dead birds sent to New Brunswick by their owner at Merrick, N. Y. He reported that the death rate at his farm had been consistently low, but that suddenly an increase had taken place.

The poultry plant was found to consist of three widely separated houses, well-kept and clean, large and airy. One entire side of each unit was enclosed by glass. The diet was carefully supervised and contained an abundance of sprouted oats and cod liver oil.

House I, 92 x 16 ft., contained 425 White Leghorn pullets, December, 1927. Cholera then appeared and gave rise to a relatively low constant death rate. By March, about 35 per cent of the population had succumbed. The entire population of 341 individuals was tested

TABLE I

Incidence of P. avicida Carriers and Occurrence of Localized Lesions at Merrick, Long Island

House	Date	Number of fowl examined	Per cent carriers	Nasal catarrh	Canker	Roup	Swollen wattles
II	Jan. 25 '28	22	45	0	0	0	0
I	Jan. 28	341	11	25	0	1	13
I	May 10	90	1	9	0	1	1

for carriers late in January, 1928. Thirty-seven or 11 per cent were positive, and note was made of a high incidence of local infection (Table I). Late in March, the survivors were combined with a similar group from House III. In May, 1928, a sample of 90 birds was tested for carriers. Only one was positive, although six of the sample were known to have been carriers at the previous testing in January. The physical condition of the birds also seemed much improved (Table I).

House II, 100 x 20 ft., housing 700 White Leghorn pullets, suffered the worst outbreak. The disease appeared here also in December, 1927, but in a more severe form. In four weeks 45 per cent of the population had succumbed. Losses then ceased abruptly. Late in January, when a "sample" of twenty-two birds was tested for carriers, 10 or 45 per cent were positive, but no local infections were noted.

House III was divided into three compartments. The first, 65 x 22 ft., housed 400 White Leghorn hens, the second, 50 x 22 ft., contained 300 similar hens. These two populations remained free of cholera. The remaining end compartment, 40 x 22 ft., housed 300 White Leghorn pullets, similar to those occupying Houses I and II. These birds began to die after mortality in Houses I and II had practically ceased, and by late spring the total deaths were estimated to be 37 per cent. No carrier determinations were made at the time of the epidemic, but the tested sample of survivors from Houses I and III showed a very low rate.

The total mortality in this flock can merely be estimated. The owner stated that 1325 pullets were added to his stock in the summer preceding the epidemic. Of these, 525 were known to have died during the winter of 1928. Forty-seven were received at the laboratory for autopsy; all but one yielded pure cultures of *P. avicida*. It was presumed, therefore, that the deaths were in the main due to *P. avicida* infection.

Of the strains of *P. avicida* recovered, three were of the "fluorescent" and 64 the "intermediate" colony type. No "blue" colony forms were obtained. The three "fluorescent" colony strains came from the blood of fatal cases in House II at the height of the epidemic. The range of acid agglutination was tested for 48 of the strains plus three "blue" colony variants obtained from the "fluorescent" forms. The results are given in Table II. The three "fluorescent" strains showed the typical narrow zone of flocculation, pH 2.4–3.0; the "blue" variants, the usual wide zone, pH 2.4–5.4, and the "intermediates," a scattered range between the limits, tending toward the more acid range. No distinction between "autopsy" and "carrier" strains was apparent.

Agglutination tests were made with "Pa" antisera (1 a) diluted 1:100. All save the three "fluorescent" colony forms showed flocculation. The agglutination titre of twenty-eight "intermediate" plus the three "blue" variants from the "fluorescent" strains was then determined (Table III). One "blue" variant agglutinated to 1:640; the others approximately to titre. Agglutination of the intermediates ranged from 1:160 to 1:2500; the greater number fell in the range 1:640–1:1280.

The virulence of fourteen autopsy strains and nineteen carrier strains was tested according to the method described in the second paper of this series (1 b). Twenty birds were used for the tests above, for each strain. The results are recorded in Tables IV and V. Several of the autopsy strains were titrated repeatedly. Hence the data on these strains are considered to be the more accurate index of virulence. The average mortality of all autopsy strains was 33 per cent.

TABLE II

Zone of Acid Agglutination. P. avicida Strains from Merrick, N. Y.

Colony type	Zone of agglutination pH range	Number of strains
Fluorescent	2.4 only	1
	2.4–3.0	1
	3.0 only	1
Blue variant	2.4–5.0	2
	2.4–5.4	1
Intermediate	2.4–4.2	2
	2.4–5.4	4
	2.5–3.4	2
	2.5–3.6	3
	2.5–4.1	3
	2.5–4.4	4
	2.5–4.7	3
	2.5–5.1	5
	3.0–3.5	1
	3.0–4.2	1
	3.0–5.0	9
	3.0–5.2	2
	3.0–5.4	1
	3.2–3.5	1
	3.4–4.7	3
	Spontaneous	1

TABLE III

Reaction with "Pa" Type Serum. P. avicida Strains from Merrick, N. Y.

Colony form	Agglutination Maximum serum dilution	Number reacting
Fluorescent	None	3
Blue variant	1:640	1
	1:1280	1
	1:2500	1
Intermediate	1:160	1
	1:320	2
	1:640	7
	1:1280	10
	1:2500	8

TABLE IV

Virulence of Merrick "Autopsy" Strains of P. avicida

Culture	Type	Isolated	Tested	Per cent dead	
629	Fl.	Jan. 22	Jan. 23	20	
			Feb. 15	20	
			Mar. 6	10	av. 17
630	Int.	Jan. 22	Jan. 23	15	
			Mar. 8	20	av. 17
631	Fl.	Jan. 22	Jan. 23	35	
			Feb. 8	40	
			Mar. 8	40	
			Apr. 11	60	av. 44
635	Int.	Jan. 30	Mar. 8	30	
638	Fl.	Jan. 30	Feb. 15 ('28)	60	
			Mar. 8	50	
			Mar. 21	40	
			Apr. 18	30	
			Apr. 25	40	
			Mar. 6 ('29)	30	av. 40
641	Int.	Jan. 30	Mar. 6	20	
642	Int.	Jan. 30	Mar. 6	60	
			Apr. 5	30	av. 45
644	Int.	Jan. 30	Mar. 6	20	
645	Int.	Feb. 1	Feb. 15	50	
			Feb. 20	60	av. 55
647	Int.	Feb. 1	Mar. 6	20	
648	Int.	Feb. 1	Mar. 6	40	
650	Int.	Feb. 1	Feb. 15	30	
			Feb. 20	50	av. 40
651	Int.	Feb. 1	Mar. 8	50	
702	Int.	Feb. 6	Mar. 6	20	
Average.....				33%	

In these titrations no significant difference in the killing power of "fluorescent" and "intermediate" colony strains was apparent. The average mortality of all "carrier" strain titrations was 21 per cent. This figure is lower than that of the "autopsy" strains, but since only one titration of each was made, the difference is of questionable significance.

The interpretation of these studies is necessarily limited. However, the following summary includes the salient facts. An epidemic of fowl

TABLE V

Virulence of Merrick "Carrier" Strains of P. avicida

Culture	Type	Isolated	Tested	Per cent dead
687	Intermediate	January 28	February 20	20
660	"	" 28	April 5	0
690	"	" 28	" 5	0
654	"	" 22	" 11	50
655	"	" 22	" 11	0
657	"	" 22	" 11	20
658	"	" 22	" 11	50
653	"	" 22	" 18	20
659	"	" 22	" 18	20
661	"	" 22	" 18	30
663	"	" 22	" 18	20
664	"	" 22	" 18	10
665	"	" 22	May 2	0
666	"	" 22	" 2	30
667	"	" 22	" 2	40
668	"	" 22	" 2	30
669	"	" 22	" 2	30
670	"	" 22	" 2	10
672	"	" 22	" 2	40
Average.....				21

cholera arose during the winter in a healthy flock of birds. Its mode of onset was not determined, and the owner stated that his birds had previously been free of the disease. The epidemic ran different courses in three population units and failed to appear in a fourth group of hens. In one infected group, the epidemic took an explosive form, with high, wave-like mortality over a brief period. Few cases of localized infection developed. In two other groups, the epidemic smoldered with low, relatively constant mortality rate over a period of

months. Strains of *P. avicida* from the two populations examined were indistinguishable. Following the epidemics, carriers were very few in number.

From these facts, it seems probable that "epidemic" strains of *P. avicida* were introduced into the flock from some outside source. Apparently, the birds possessed no specific immunity to the infection, but those in House I may have been more resistant than the population of House II. The crowding in House II, by decreasing the resistance of the inhabitants and increasing the risk of infection, may have been responsible for the explosive type of attack. Finally, the fact that few post-epidemic carriers were encountered indicates that, as in similar "D type" rabbit infections (1 c), "epidemic" strains of *P. avicida* are relatively non-vegetative.

B. Study of Endemic P. avicida Infection

A similar investigation was made at a small farm in Deans, N. J., consisting of 45 White Leghorn hens, cared for entirely by the owner. The pen was very dirty and neglected, dark, and in an exposed location. Some sort of epidemic of fowl cholera was known to have occurred in previous years, and the birds under observation were known to be survivors.

Five trips were made to the farm, November, 1927 to March, 1928. A census was taken, the clinical condition of the birds noted, and cultures obtained from the nasal passages of each. The birds were tagged, so that a four months' record of each was obtained. The results of these investigations are given in Table VI.

No birds died within the period. Nineteen showed local upper respiratory infections—roup, canker, mucous discharge, wattle disease—at one or more examinations. Thirty-one proved to be carriers of *P. avicida* on at least one test. The carrier rates at each examination were 9.7, 40.5, 16.2, 31.5, and 29.0 per cent.

Fifty-two strains of *P. avicida* were recovered from this flock. Apparently, all formed "blue" colonies (1 a) on the first transfer. Twenty-seven were titrated in "Pa" antiserum (1 a). The results are shown in Table VII. Two did not agglutinate; two showed spontaneous agglutination; three reacted within the range 1:32–1:128; four agglutinated at a dilution of 1:256; fourteen to 1:152, and two to 1:1024 and 1:2048 respectively.

TABLE VI

Results of Serial Examinations for P. avicida Carriers, at Deans, N. J.

Bird	November 23	December 17	January 17	February 7	March 23
1	0	0 Canker	0	−	+
2	0	0 Mucus	0	0	0
3	−	0	−	0	+
4	+ Canker	0	0	0	+
5	0	+ Mucus	0	0	0
6	0	+	0	0	+
7	0 Membrane	−	0	+ Canker	0
8	0	0 Canker	0	+	−
9	0	0 Mucus	0	0 Roup	−
10	0	0	0	0	0
11	0	0 Mucus	0	+	0
12	+	+ Mucus	0	0	−
13	0	+ Wattle	0	0 Mucus	0
14	0	+ Canker	0	+	+
15	0	0	0	0	0
16	0	0	0	0	0
17	0	+	0	−	0
18	0	+ Mucus	0	+ Mucus	0 Roup
19	+	+	+	0	+
20	−	0 Mucus	0	+	0
21	0	+	0	0	0
22	0	0	0	0 Mucus	0
23	0	+ Canker	0	0 Roup	0
24	0	−	0	0 Roup	−
25	0	0	+	0	0
26	0	0	0	0	0
27	+	0	0	+	0
28	0	+	+	+	+
29	0	0	0	0	0
30	0	0	0	0	0
31	0	0	0	0 Canker	0
32	0	+	0	0	−
33	0	+	0	0	0
34	0	+	+	0	0
35	0	0	0	+	0
36	0	0 Mucus	0	0 Mucus	0
37	0	0	+	+	0
38	0	0	0	+	+
39	0	0	0	0	−
40	0	+ Mucus	0	−	0
41	0	0	0	0	0
42	0	0	0	0	+
43	−	+	0	−	+
44	0	+	+	+ Roup	+
45	−	−	−	+	−
Positive	4 = 9.7%	17 = 40.5%	6 = 16.2%	13 = 31.5%	11 = 29%
Negative	37	25	37	28	27
Not done	4	3	2	4	7

0 = Negative; + = *P. avicida* recovered; − = No examination.

Acid agglutination tests, made on twenty-nine strains, are given in Table VIII. Twenty-four showed the wide zone characteristic of "blue" colony forms, but five came down well toward the acid side, pH 2.4–4.0

This study may be summarized as follows: in a small flock, known to have survived cholera infection the previous year, the endemic manifestation of the disease was studied. No deaths occurred, but the per cent of local lesions and nasal carriers increased during the winter

TABLE VII

Reaction with "Pa" Serum. P. avicida Strains from Deans, N. J.

Serum dilution	Number of strains reacting	Serum dilution	Number of strains reacting
No reaction	2	1:256	4
Spontaneous	2	1:512	14
1:32	1	1:1024	1
1:64	1	1:2048	1
1:128	1		

TABLE VIII

Zone of Acid Agglutination. P. avicida Strains from Deans, N. J.

Zone of agglutination pH range	Number of strains	Zone of agglutination pH range	Number of strains
2.4 only	1	2.4–4.6	4
2.5–3.0	1	2.4–5.0	1
2.4–3.2	1	2.4–5.4	6
2.4–3.5	1	3.0–4.2	1
2.4–4.0	1	3.0–4.6	3
2.4–4.2	5	3.0–5.4	4

months. Apparently the birds differed in their ability to resist infection, for in spite of the evidence of spread of the organisms, some birds remained free throughout. All, however, were probably more than normally resistant, for they were survivors of a previous epidemic. The organisms recovered possessed the general characteristics of the "blue" colony form, although a few resembled the "intermediates" in their behavior. Unlike the "epidemic" fluorescent and intermediate colony forms, they exhibited the power of vegetation and spreading in the community. In this respect they were similar to the "mucoid" strains of *P. lepiseptica* (1 c).

SUMMARY

Field studies of fowl cholera on two commercial poultry farms are described. One farm, previously free of cholera, was studied during an active epidemic, which occurred during the winter months. The strains of *P. avicida* recovered, both from "autopsy" and from "healthy carriers" proved generally similar, and to be of the "fluorescent" or "intermediate" colony type, which is of relatively high virulence. After the subsidence of the epidemic, these strains tended to disappear.

The second flock consisted of a small group of birds which had survived an epidemic of cholera the previous year, and in which the infection was prevailing in endemic form. No deaths occurred during the period of observation, but the number of birds with localized lesions and the number of carriers increased to a high level during the winter months. The strains of *P. avicida* were apparently of the "blue" colony form, although some, as shown by their acid and serum agglutination reactions, resembled the "intermediates." These strains appeared to be spreading rather than dying out. The individual fowls differed in their response to the presence of infection; some showed localized lesions, others were carriers, while still others seemed entirely refractory.

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THE EPIDEMIOLOGY OF FOWL CHOLERA

V. Further Field Observations of the Spontaneous Disease

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(Received for publication, October 31, 1929)

Field studies of the sort described in the previous paper (1b) were made on one additional poultry farm at Belle Meade, N. J., where fowl cholera was known to have prevailed in endemic form. The observations at this farm were better controlled, more detailed, and extended over a longer period of time. The purpose of this study was twofold: 1) To gain further knowledge of endemic cholera, the common, almost ubiquitous form of the disease in this locality, and 2) To test a method of control. The results obtained are described in this paper.

1926–1927

The flock at Belle Meade consisted of some 2000 White Leghorn chickens, maintained for commercial purposes. Winter outbreaks of fowl cholera were known to have occurred in 1925–1926. The farm was of modern construction and well-kept, comprising a long building with subdivisions, several outbuildings, and unrestricted "runs." Windows extended along one entire side of the large building. Details of the floor plan are given in Text-Figure 1.

House I contained 239 pullets. Seven tests for carriers were made between March 4 and May 17, 1927, on random samples of four to eighty-four birds (Table I). No carriers were detected, but some individuals were not examined. Twenty-two birds died in this house from December, 1926 to June, 1927. All were autopsied and four showed *P. avicida* infection (Table II).

House II contained 273 pullets. Six tests for carriers were made from March 4 to May 4, 1927, during which time each bird received at least one examination (Table I). No carriers were found. Eleven deaths occurred during the period December, 1926—June, 1927. They were autopsied, but proved negative for *P. avicida* infection (Table II).

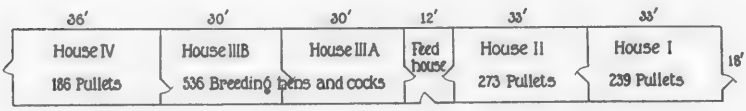
House III was divided into two compartments prior to January 1, 1927. One compartment contained pullets; the other, hens which had survived a fowl cholera

epidemic the previous winter. In January, the pullets were removed to House IV, the partition removed, and a number of cockerels added. This group of hens and cockerels composed the breeding stock.

The entire group of 536 was tested for carriers during the period January–March, 1927, the greater number receiving the examinations (Table I). Seventy-four carriers of *P. avicida* were found. Eighty-four deaths occurred during the year, of which sixty-one were shown at autopsy to be due to fowl cholera (Table II).

House IV contained 186 pullets, 85 of which were transferred from House III. All were tested at least once, and most of them twice. Ten carriers of *P. avicida* were found (Table I). During the year twenty-five died and were autopsied. Eighteen had fowl cholera and yielded positive cultures (Table II).

These observations permit a tentative explanation of the mode of spread of the disease during the year. In the first place, the infection was brought to the flock, in all probability, by the hens in House III,



TEXT-FIGURE 1

themselves survivors of an outbreak the year before, and known to have been infected early in the fall (Table I). Its spread was brought about by the pullets in House III, which were later transferred to House IV, and by way of an occasional straggler gaining access to Houses I and II. The infection assumed a seasonal character, but the septicemic form of the disease and the carrier state increased in amount during the winter months, and practically disappeared during the summer (Text-Figure 2). Finally, there proved to be a distinct correlation between the number of *P. avicida* carriers and the number of deaths from fowl cholera. In House II, for example, where each bird was examined and found negative for *P. avicida*, there was no cholera mortality. In House I, where the carrier incidence, if any, must have been low, specific deaths were low. In Houses III and IV, on the contrary, both the carrier incidence and cholera death rate were high, the latter representing the greater part of the total mortality in these houses (Text-Figure 3).

TABLE I

Carrier Tests on "Belle Meade" Poultry Flock, 1926–1927

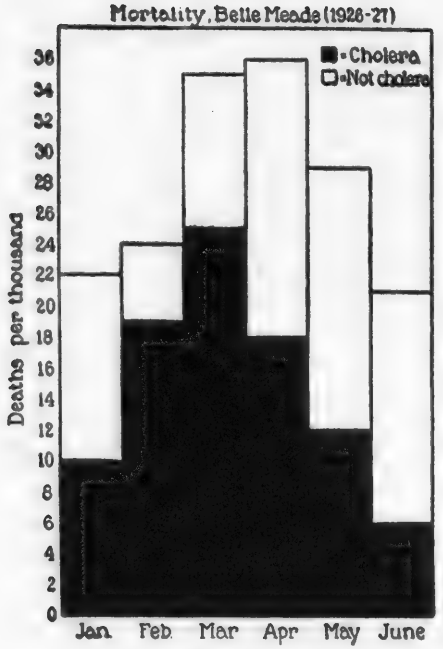
Date	Number of birds examined				Carrier percentage			
	House I 239 birds	House II 273 birds	House III 536 birds	House IV 186 birds	House I	House II	House III	House IV
December 1	0	0	9	90	—	—	0.0	0.0
January 5	0	0	222	3	—	—	6.3	0.0
January 11	0	0	213	0	—	—	5.3	—
January 24	0	0	212	4	—	—	20.3	0.0
January 31	0	0	223	2	—	—	0.4	0.0
February 7	0	0	119	0	—	—	5.1	—
March 4	38	36	9	42	0.0	0.0	2.4	0.0
March 14	34	56	0	47	0.0	0.0	—	4.2
March 29	67	38	0	45	0.0	0.0	—	11.0
April 12	84	23	0	0	0.0	0.0	—	—
April 26	80	48	0	0	0.0	0.0	—	—
May 4	4	67	0	45	0.0	0.0	—	6.6
May 17	15	0	0	0	0.0	—	—	—
June 3	0	8	0	0	—	0.0	—	—
Total.....	322	276	1007	278				

0 = No test.
— = No test.
0.0 = Zero per cent carriers.

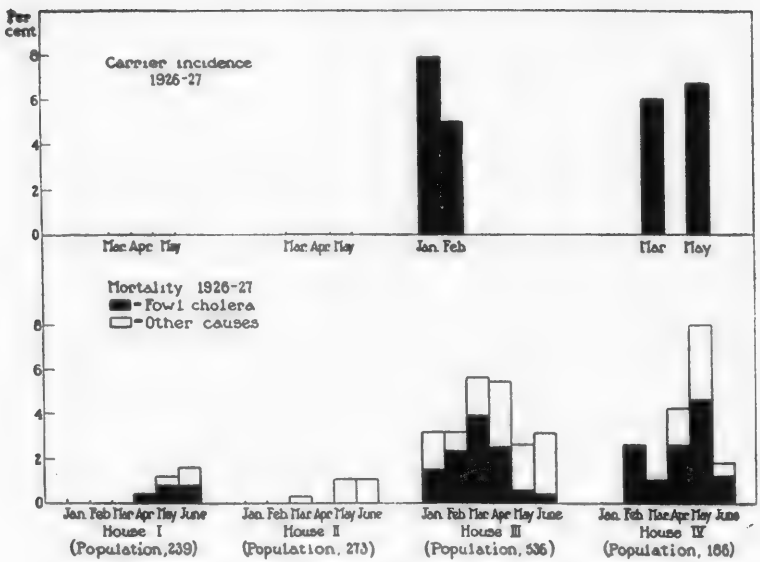
TABLE II

Mortality of "Belle Meade" Poultry by Months, 1926–1927

Month	House I		House II		House III		House IV	
	Fowl cholera	Other causes	Fowl cholera	Other causes	Fowl cholera	Other causes	Fowl cholera	Other causes
December	0	0	0	0	0	0	0	0
January	0	0	0	0	8	9	0	0
February	0	0	0	0	12	4	5	0
March	0	0	0	1	20	9	2	0
April	1	0	0	0	12	14	5	3
May	2	1	0	3	3	9	8	5
June	1	1	0	3	2	12	2	1



TEXT-FIGURE 2



TEXT-FIGURE 3

1927–1928

During the second year, the work was carried on as before, except that an attempt was made to remove all carriers from the breeding population and test the effect of this procedure on the amount of fowl cholera. As a preparation for this

TABLE III

Results of Carrier Tests; Population A, Belle Meade, N. J., 1927–1928
Population = 396

Date	Number examined	Number of carriers	Per cent carriers
11/ 2/27	31	0	
9	196	0	0.0
30	30	0	
12/14	28	0	
21	21	0	0.0
29	60	0	
1/ 8/28	60	0	0.0
2/29	50	0	0.0
3/21	66	0	0.0
4/11	54	0	0.0
5/ 2	139	0	0.0

TABLE IV

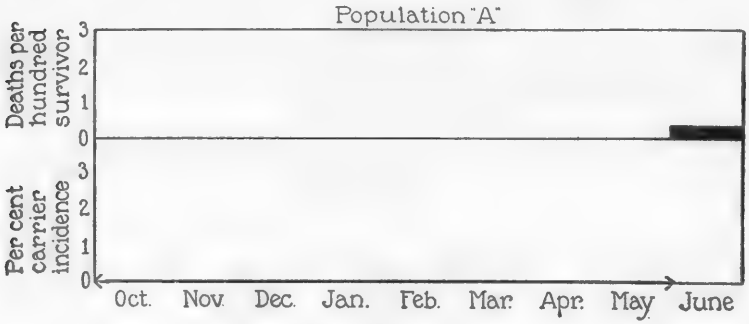
Results of Carrier Tests; Population B, Belle Meade, N. J., 1927–1928
Population = 714

Date	Number examined	Number of carriers	Per cent carriers	
12/21/27	196	0	0.0	
1/ 5	183	0	0.0	0.0
1/18/28	117	0	0.0	
2/29	98	0	0.0	
3/28	76	1	1.3	
4/18	50	10	20	18
4/25	118	19	16	
5/23	119	21	18.5	

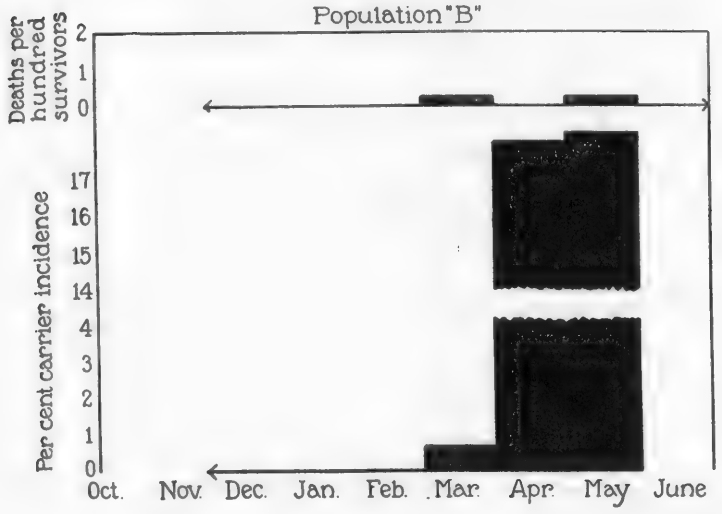
test, a new house with three compartments was constructed and the entire plant thoroughly cleaned and disinfected. As a result, there occurred considerable shifting of groups. For the purpose of this discussion, however, the entire population can be considered as composed of three distinct divisions, A, B, and C.

Population A. Consisted of 396 pullets, offspring of hens and cockerels of

House III of the previous year. These birds were born and raised in confinement and hence were supposed to have had no contact with other birds. They were kept in Houses I and II. A representative sample was examined at monthly



TEXT-FIGURE 4



TEXT-FIGURE 5

intervals from November, 1927 to May, 1928 inclusive. No carriers of *P. avicida* were detected (Table III). During this period, one bird died of fowl cholera (Table VI, Text-Figure 4).

TABLE V

Results of Carrier Tests; Population C, Belle Meade, N. J., 1927–1928

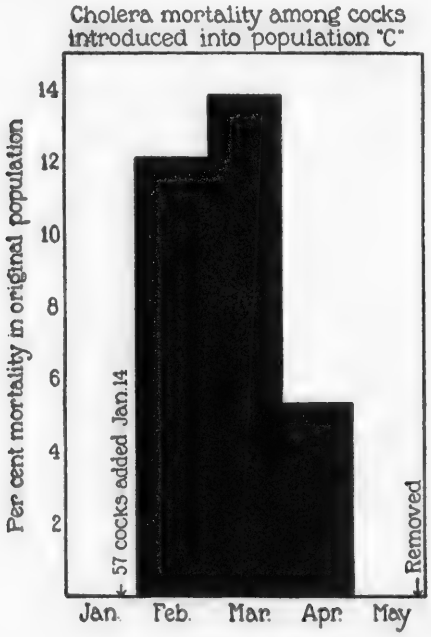
Date	Number examined	Number of carriers	Per cent carriers	Average
10/20/27	95	7	7.4	7.3
10/26	138	10	7.2	
11/ 2	200	23	11.5	4.5
11/16	142	3	2.1	
11/18	74	1	1.3	
11/30	214	7	3.2	
12/ 7	160	5	3.1	2.5
12/14	197	4	2.0	
2/ 1/28	159	4	2.5	2.8
2/ 7	314	11	3.5	
2/16	240	6	2.5	
3/ 7	227	8	3.5	5.1
3/14	207	21	9.9	
3/21	117	5	4.3	
3/28	107	3	2.8	
4/ 4	89	1	1.1	0.7
4/11	106	0	0.0	
4/18	92	1	1.1	
5/16	122	4		3.2

TABLE VI

Mortality—Belle Meade, N. J., 1927–1928

Month	Cholera				Non-cholera	Total
	Pop. A (396)	Pop. B (714)	Pop. C (653)	Unknown		
October	0	0	0	0	0	0
November	0	0	4	0	6	10
December	0	0	2	1	44	47
January	0	0	1	0	23	24
February	0	0	3 Females 7 Males	0	17	27
March	0	1	9 Females 8 Males	0	10	28
April	0	0	0 Females 3 Males	1 Female 1 Male	24	29
May	0	1	3	0	18	22
June	1	0	0	0	3	4

Population B. Consisted of 714 pullets similar to those in Population A, save that they were allowed to run about the farm at will until December, 1927. They were then placed in Houses III and IV. A sample lot was tested each month for carriers (Table IV). None was found until March, 1928. At that point, 1.3 per cent of the sample of 76 proved positive; in April 18 per cent of 168 birds tested, and in May 18.5 per cent of 119 so examined. In spite of this, only two deaths from cholera occurred during the year, one in March and one in May (Table VI, Text-Figure 5).



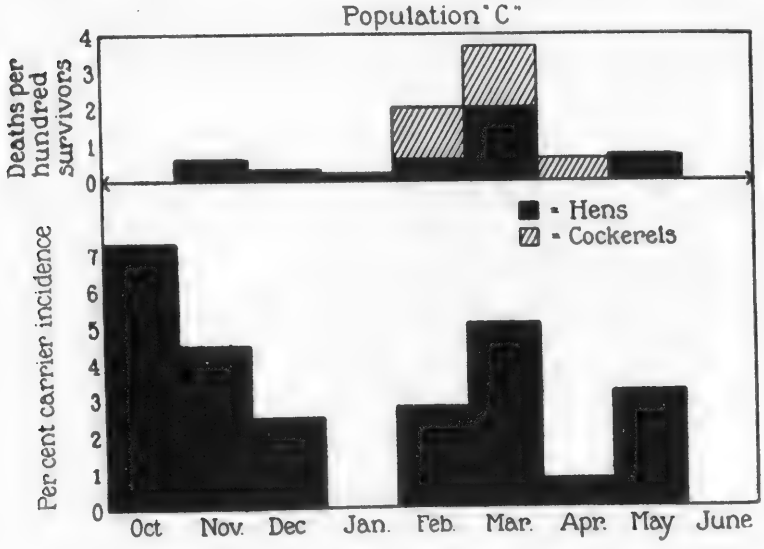
TEXT-FIGURE 6

Population C. Comprised the breeding hens selected on the basis of high egg production from the pullets of Houses I, II, and IV. Fifty-seven cockerels, previously shown to have been free of *P. avicida*, were added in January, 1928. The effort to remove all carriers from this group was continued, but at best, seven days elapsed between test and elimination.

Tests for carriers were made from October, 1927 to May, 1928. Each bird had received from two to four examinations. The results, given in Table V, indicate that the population was never free of carriers, in spite of the removal of positive

cases after each test. Nevertheless, the rate was low, and declined progressively as the number of tests increased, until March, 1928, when, coincident with the rise in number of carriers in Population B, an increase in carrier incidence occurred. Subsequently, in April and May, it declined again.

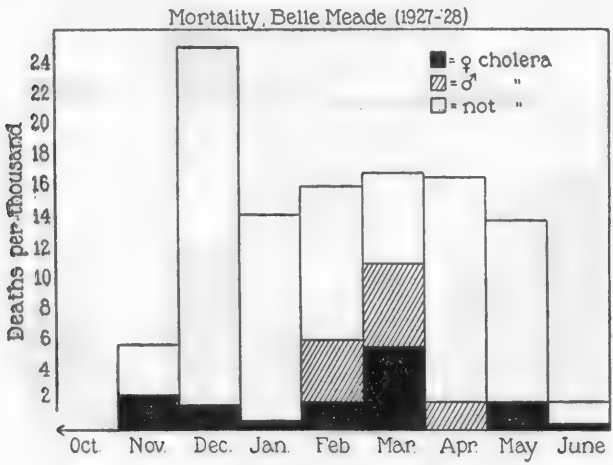
The mortality in Population C is given in Table VI. Four cholera deaths occurred in November, two in December, and one in January. In February three hens and seven cockerels died of cholera, in March nine hens and eight cocks, April three cocks, and May a total of three (Text-Figures 6 and 7).



TEXT-FIGURE 7

This series of observations during 1927–1928, together with those made during the previous year, suggests further conclusions as to the mode of spread of endemic fowl cholera. In the first place, it appears probable that the infection survived over the summer in "healthy" carrier pullets and hens and was spread by them during the following winter throughout the new breeding stock. Thus, the breeding stock C was known to be infected at the outset, whereas the pullets, A and B, were not. Spread took place among the breeders, C, then to some of the pullets, B, presumably because of some technical error. However, no infection took place among the well-protected group of pul-

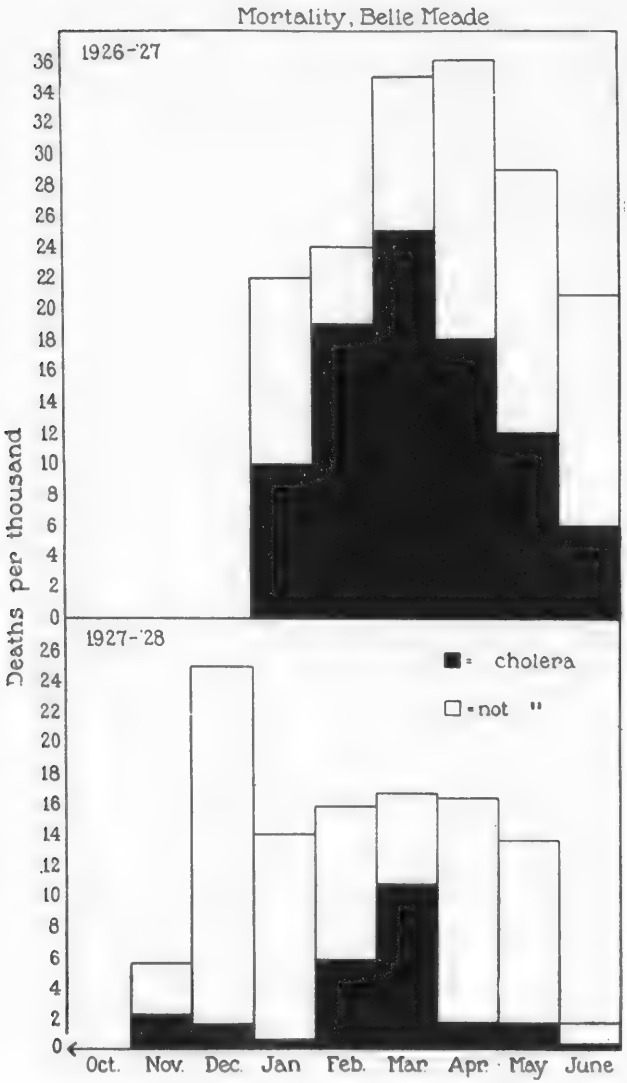
lets, A. In the next place, it seems that the infection may assume the form of an epidemic when susceptible immigrants are brought into the community. This was noted when cocks were added to the group of hens designated Population C. Further evidence was forthcoming to indicate the determining rôle of host resistance in the spread of infection in that Population B, although exposed to a considerable risk, did not suffer an epidemic. The sudden increase in the number of carriers in Population B during March and April, without an accompanying rise in number of fatalities, can be explained at present only



TEXT-FIGURE 8

by assuming that the resistance of the community was enhanced by the seasonal influences of spring.

Three additional points remain to be considered. First, the amount of *P. avicida* infection in this community was seen to vary according to season in 1927–1928 as in 1926–1927. During both years, the amount of carrier infection and fatal infection reached its maximum in winter and minimum in summer (Text-Figure 9). Second, the number of cholera deaths was observed usually to vary directly with the number of *P. avicida* carriers. In Population A, for example, there were no cases of carrier infection and no fatal cholera cases.



TEXT-FIGURE 9

In Population C, carrier rate and cholera mortality rate varied together. Both decreased progressively from October to January and then increased during February and March. In Population B, however, this relationship was marked. Presumably the spring increase in resistance of the fowl limited the infection to the carrier state.

The third point to be discussed is the effect of removing *P. avicida* carriers from Population C. Unfortunately, technical and practical difficulties prevented the completion of a critical test with suitable control. It may be noted, however, that in spite of the fact that severe outbreaks of cholera had occurred each winter for the past several years, no such epidemic occurred in 1927–1928. Indeed, the cholera mortality was less than one-fifth that of the previous year (Text-Figure 9). The carrier rates as well show this marked decrease. In 1927–1928, cholera was confined almost entirely to the breeding Population C, and in this community a comparison of carrier rate and mortality rate figures for the years 1926–1927 and 1927–1928 demonstrates the marked limitation of the infection. There is some reason, therefore, to believe that the spread of fowl cholera may be controlled by the elimination of carriers.

Bacteriology

Strains of *P. avicida* recovered from the Belle Meade flock during 1926–1927 and 1927–1928 were studied and compared. The results of these tests are as follows: the 1926–1927 strains were apparently all of the "blue" colony form (1a), the 1927–1928 strains "blue," except for an occasional culture with the narrow acid agglutination zone and low antiserum agglutination titre characteristic of the "intermediate" colony form (1a).

The acid agglutination zone of thirty-three 1926–1927 strains and thirty-one 1927–1928 strains was determined. The results are given in Table VII. The 1926–1927 strains, with one exception, showed the characteristic "blue" colony type of reaction, agglutination from pH 2.4 to 5.4, while four of the 1927–1928 strains reacted only in the more acid mixtures. No distribution of cultures on the basis of time, or place of isolation was evident. Carrier and autopsy strains behaved in a similar manner.

All strains were tested in "Pa" serum diluted 1:100 (1a). All

1926–1927 cultures were agglutinated strongly; eleven obtained in 1927–1928 failed to agglutinate. Beside this they showed a narrow

TABLE VII

Zones of Acid Agglutination of Strains of P. avicida Obtained from Carriers and Cases of Fowl Cholera at Belle Meade, N. J.

Agglutination zone	Number of strains reacting	
	1926–1927	1927–1928
None	0	4
pH 2.4–3.2	1	1
2.4–3.5	0	1
2.4–4.0	0	1
2.4–4.2	0	1
2.4–5.0	0	6
2.4–5.3	0	4
2.4–5.4	21	0
2.5–3.5	1	0
2.5–5.0	1	0
3.0–3.5	1	0
3.0–4.0	2	0
3.0–4.2	1	6
3.0–5.0	1	4
3.0–5.4	3	1
3.0–5.9	0	2
3.2–3.5	1	0
Total number.....	33	31

TABLE VIII

Agglutination Titre in "Pa" Antiserum of Strains of P. avicida Obtained from Carriers and Cases of Fowl Cholera at Belle Meade, N. J.

Serum dilution titre	Number of strains agglutinated	
	1926–1927	1927–1928
1:250	—	7
1:500	3	15
1:1000	9	43
1:2000	7	2
1:4000	7	—

zone of reactivity in acid buffer. The titre of twenty-six 1926–1927 strains and sixty-seven 1927–1928 strains was tested in "Pa" serum

(Table VIII). Of the cultures obtained the first year, three agglutinated to a dilution of 1:500, nine to 1:1000, seven to 1:2000, and

TABLE IX

Virulence of Carrier and Autopsy Strains, 1927–1928

Strain	Source	Isolated	Tested	Per cent dead
695	Autopsy	2/ 3/28	2/ 8/28	0
696	"	1/ 3/28	2/ 8/28	0
714	Carrier	2/16/28	2/20/28	0
715	"	2/16/28	2/20/28	0
717	"	2/16/28	2/20/28	0
718	"	2/16/28	2/20/28	0
728	Autopsy	3/ 7/28	3/13/28	10
729	"	3/ 7/28	3/13/28	0
730	"	3/ 7/28	3/13/28	0
733	"	3/ 7/28	3/13/28	0
745	Carrier	3/14/28	3/21/28	0
746	"	3/14/28	3/21/28	0
747	"	3/14/28	3/21/28	0
748	"	3/14/28	3/21/28	0
749	"	3/14/28	3/21/28	0
750	"	3/14/28	3/21/28	0
751	"	3/14/28	3/21/28	0
752	"	3/14/28	3/21/28	0
770	"	3/14/28	3/28/28	10
779	Autopsy	3/14/28	3/28/28	0
781	"	3/14/28	3/28/28	20
783	Carrier	3/28/28	3/28/28	0
784	Autopsy	3/14/28	3/28/28	20
785	"	3/14/28	3/28/28	10
792	"	3/29/28	4/ 5/28	0
793	"	3/29/28	4/ 5/28	0
794	Carrier	3/28/28	4/18/28, 4/25/28	0
795	"	3/28/28	4/18/28, 4/25/28	0
796	Autopsy	3/28/28	4/26/28	0
798	"	3/29/28	4/19/28, 4/26/28	0
801	Carrier	4/18/28	5/ 2/28	0
805	"	4/18/28	5/ 2/28	0
813	Autopsy	4/18/28	5/ 2/28	0

Average mortality: Carrier strains..... 0.6%
Autopsy strains..... 4.0%

seven to 1:4000, while of those received the second year, seven agglutinated in a dilution of 1:250, fifteen to 1:500, forty-three to

1:1000 and two to 1:2000. Three additional sera were prepared for 1927–1928 strains. Two of these strains agglutinated to titre in "Pa" serum; the other, not at all. The three antisera made from these cultures agglutinated the "Pa" strain, and their specific agglutinins were absorbed by "Pa" antigen. Conversely, "Pa" agglutinins were absorbed from the "Pa" serum by the three 1927–1928 antigens. Hence, no serological differences in these strains could be proved.

The virulence of a number of 1926–1927 strains was tested by the method previously described (1a). None of them killed. The following year, twenty carrier strains and sixteen autopsy strains from Population C were tested. A very low mortality resulted (Table IX). The titrations indicate that the virulence of all strains was similar; 1926–1927 strains were similar in this respect to 1927–1928 cultures, "carrier" strains similar to "autopsy" strains, and strains from houses with relatively little cholera the same as those from populations where cholera was most frequent.

SUMMARY

An investigation of endemic fowl cholera, the common form of the disease in this locality, has been made at a poultry farm in Belle Meade, N. J. The focus or reservoir of *P. avicida* proved to be the healthy pullets which had become carriers the previous year and which were selected as the breeding stock for the ensuing season. From these carriers, the organisms spread and gave rise during the winter months to the various forms of infection, including the carrier state, localized upper respiratory disease, and typical cholera. Strains of *P. avicida* were in general very similar and of the "blue" colony type. They were all of the same low degree of virulence; no differences were demonstrable between a) "carrier" and "autopsy" strains, b) strains where the infection was spreading and severe, and strains where disease was rare, and c) "autumn" and "winter" strains. In general, a relatively high carrier rate was accompanied by a high mortality rate, although in one instance, a community with high carrier incidence plus a probable high degree of host resistance suffered but little fatal infection. An attempt was made to reduce the amount of cholera by the removal of carriers. The results of this measure indicate that such a procedure is both effective and practical.

The authors desire to express thanks to Mr. C. B. Hudson for his valuable assistance in this work, and to Mr. Max Bogner, the owner of the Belle Meade Farm for his cooperation and aid.

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EARLY PULMONARY LESIONS IN PARTIALLY IMMUNE ALCOHOLIZED MICE FOLLOWING INHALATION OF VIRULENT PNEUMOCOCCI

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(Received for publication, November 11, 1929)

In preceding papers (1) it has been shown that virulent pneumococci generally disappear from the lungs of normal mice within a few hours after inhalation and do not give rise to generalized infection. In alcohol-intoxicated mice, however, pneumococci persist in the lungs for a longer period and a fatal septicemia frequently follows. It has further been shown (2) that a high degree of immunity is produced in mice following repeated inhalations of live pneumococci and that a less marked immunity results from exposure to a spray of dead organisms. In the instances in which non-immune mice succumb as the result of infection following a pneumococcus spraying while alcoholized, death is due to general septicemia and there is no evidence of localized infection in the lung. However, mice which have been rendered partially immune by inhalation of live or killed pneumococci and are later alcoholized and exposed to virulent pneumococci show definite evidence of localized pulmonary infection.

In the foregoing experiments only animals which had succumbed to the pneumococcus infection were studied. Furthermore, the entire lung was not examined. There was the possibility that:—(1) some mice might have developed pneumonia but subsequently recovered, and (2) that early pneumonic lesions might have been missed as all the lobes were not examined histologically. In order to accurately determine the incidence of pulmonary localisation and to detect the initial pulmonary reactions in partially immunized mice following inhalation of virulent pneumococci while alcoholized, the following experiments were undertaken. This paper is a report on the examination of serial sections of the lungs of 81 partially immunised mice which

were allowed to inspire virulent homologous pneumococci while alcoholized.

Method

The mice to be immunized were placed in the spray chamber previously described and sprayed at intervals of 2 to 3 days with broth cultures of pneumococci which had been killed by heating to 60° for 30 minutes, or heat killed pneumococci suspended in salt solution.

Ten days after the last spraying the mice were alcoholized by intraperitoneal injection of 1.5 c.c. of a 10% solution of alcohol in saline. 1 hour after the administration of the alcohol, the animals were sprayed with 50 c.c. of virulent live pneumococcus culture and allowed to remain in the spray box for 1 hour. At intervals of 6, 12, 18 and 24 hours following spraying, groups of at least 9 mice were killed by chloroform.

After the heart's blood was cultured in broth, the lungs were removed in toto and dropped into Zenker's fluid to which 5% acetic acid had been added. They were paraffin embedded and serial sections were cut 12 microns thick. Every 12th section was examined as routine, and where lesions were discovered the intervening sections were examined.

The pulmonary lesion called chronic consolidation occurs in about 3 per cent of apparently healthy stock mice. Both in the gross and microscopically the lesions are readily differentiated from lesions due to the pneumococcus.

EXPERIMENTAL

Group I. 44 mice which had been partially immunised by spraying with whole killed cultures of pneumococci were exposed to inhalations of virulent type I pneumococci while alcoholized. The incidence of pulmonary lesions and the results of the heart's blood culture of these mice are given in Table I.

From this table it is seen that of the 10 mice killed in 6 hours, the lungs were normal in 4, and congested or edematous in 5. In only one animal was there any evidence of localisation of the infection as indicated by an inflammation in the alveolar wall.

The lungs of 7 of the 10 mice sacrificed in 12 hours appeared normal; in one chronic consolidation was present, in one congestion and edema, and in one the alveolar walls were inflamed.

The lungs of 6 of the 10 mice killed in 18 hours were normal; in one chronic consolidation and in three congestion were present.

The lungs of 10 of the 14 mice killed in 24 hours were normal; in 2 chronic consolidation occurred and in 2 congestion and edema.

In none of the mice killed in 6 hours was the heart's blood culture positive and the pneumococcus was recovered from only one and two mice of those killed in 12 and 18 hours respectively. 7 or 50% of the mice killed at 24 hours, however, showed positive heart's blood cultures.

Group II. 37 mice previously partially immunised by exposure to heat killed pneumococci were intoxicated and sprayed with virulent homologous pneumococcus. The results of the blood cultures and histological examination of the lungs of these mice are given in Table II.

TABLE I

Pulmonary Lesions in Lungs of Mice Partially Immunised by Inhalations of Heat-Killed Whole Cultures of Pneumococcus Type I

Time killed after inhalation of virulent Type I pneumococcus while alcoholised

6 hours			12 hours			18 hours			24 hours		
No.	Blood culture	Pathology	No.	Blood culture	Pathology	No.	Blood culture	Pathology	No.	Blood culture	Pathology
A1	−	C	B1	−	N	C1	−	N	D1	+	C.C.
A2	−	C.E.	B2	−	Early I.I.	C2	+	C	D2	+	C.C.
A3	−	H.E., C	B3	−	C.C.	C3	−	N	D3	−	N
A4	−	Early I.I.	B4	+	C., H.E.	C4	−	N	D4	−	N
A5	−	N	B5	−	N	C5	−	N	D5	+	N
A6	−	N	B6	−	N	C6	−	N	D6	−	N
A7	−	C	B7	−	N	C7	−	C	D7	−	N
A8	−	N	B8	−	N	C8	−	C	D8	−	N
A9	−	C.E.	B9	−	N	C9	+	C.C.	D9	−	N
A10	−	N	B10	−	N	C10	−	N	D10	+	N
									D11	+	C
									D12	+	C.E.
									D13	−	N
									D14	+	N

N = normal.
C = congestion.
E = edema.
H.E. = Hemorrhagic edema.
I.I. = Interstitial inflammation i.e. lesion limited to alveolar wall.
R = Red hepatisation.
C.C. = Chronic Consolidation.
− = negative blood culture.
+ = positive blood culture.

The lungs of 6 of the 9 mice killed in 6 hours appeared normal; in 2 they were congested and edematous and in one animal there was evidence of an attempt at localisation in the form of an inflammation in the alveolar wall.

The lungs of 8 of the 9 mice chloroformed at 12 hours were normal; in one they were congested and edematous and in one chronic consolidation was present. Localised areas of red hepatisation were found in 2 of the remaining 3 animals and interstitial inflammation was present in one.

Normal lungs were found in 5 of the 9 mice sacrificed at the end of 24 hours. Chronic consolidation occurred in 2 mice, congestion and edema in one, and red hepatisation in one.

The cultures of the heart's blood of all the mice killed after 6 and 12 hours remained sterile. Positive heart's blood cultures were obtained from 2 of the animals killed after 18 hours, while the blood of 5 or 55% of the mice sacrificed after 24 hours contained pneumococci.

TABLE II

Pulmonary Lesions in Lungs of Mice Partially Immunised by Inhalations of Heat-Killed Pneumococcus Type I Vaccine

Time killed after inhalation of virulent type I pneumococcus while intoxicated

6 hours			12 hours			18 hours			24 hours		
No.	Blood culture	Pathology	No.	Blood culture	Pathology	No.	Blood culture	Pathology	No.	Blood culture	Pathology
A1	–	N	B1	–	N	C1	–	N	D1	–	C.C.
A2	–	N	B2	–	C.C.	C2	–	N	D2	–	N
A3	–	N	B3	–	N	C3	+	I.I.	D3	–	N
A4	–	N	B4	–	N	C4	–	Local R	D4	–	C.E.
A5	–	I.I.	B5	–	N	C5	–	N	D5	+	R
A6	–	N	B6	–	N	C6	–	C.C.	D6	+	N
A7	–	C.E.	B7	–	N	C7	–	N	D7	+	N
A8	–	N	B8	–	N	C8	–	N	D8	+	C.C.
A9	–	C.E.	B9	–	N	C9	+	Local R	D9	+	N
						C10	–	C.E.			

N = normal. I.I. = interstitial inflammation i.e. lesion limited to the alveolar wall.
C = congestion.
E = edema. R = red hepatisation.
– = negative blood culture.
+ = positive blood culture.

On gross examination the lungs of these 81 mice showed, in a few instances, congestion and chronic consolidation. Of these, 8 must be excluded because of the presence of chronic consolidation which would mask any slight lesions that might have been present. The lungs of 51 or 71% of the remaining mice were normal, and congestion and edema occurred in 15 or 21%. Evidence of pulmonary localisation occurred in 7 or 9%. The inflammatory process was limited to the alveolar walls in 2 mice killed after 6 hours, and in 2 animals killed after 18 and 24 hours respectively. In 3 mice exudation into the alveoli was the predominant feature and the lesions were classed as red hepatisation. Small areas of beginning con-

solidation occurred in 2 mice killed after 18 hours and the greater portion of one lobe was involved in a mouse killed in 24 hours.

Of the 81 mice, the pneumococcus was recovered from the heart's blood in 17 or 22%. Only one of the mice killed within 12 hours had a positive blood (5%), while 4 of those killed after 18 hours (20%) and 12 of the animals sacrificed after 24 hours showed a septicemia (52%). Of the 17 positive blood cultures, 4 occurred in mice with chronic consolidation and therefore must be excluded. Of the remaining 13, 6 occurred in mice with normal lungs, 4 were associated with congestion and edema, and 3 with inflammatory lesions.

Microscopic Pathology of the Early Pneumonic Lesions

This study is limited to serial sections of the 6 lungs mentioned above which showed inflammatory lesions not of the spontaneous chronic type often found in mice.

The nature of the early lesion is exemplified by the reaction occurring in the lungs of a mouse killed 6 hours after spraying:

An exudative inflammatory process involves 2 lobes, the caudal and ventral. The lesion is 2–4 m.m. wide, and does not reach the hilum nor the pleura. The inflammation is limited to the alveolar wall. The only exudation into the lumen has occurred at the junction of atria and bronchioli. Neither the large, nor small vessels nor the pleura are involved. In sections of this thickness (12 m.m.) it is not possible to say whether the thickening of the alveolar wall, with the accompanying exudate which is particularly rich in polymorphonuclear leucocytes, is due to inflammatory cells within or without the capillaries or to a combination of both. An area of atelectasis occupying about one fifth of the whole lobe is situated around the inflammation but the surrounding pulmonary tissue is congested. The area of inflammation appears to be continuous. The bronchial lymph nodes are not involved and no lymphatic involvement is apparent.

After 18 hours 2 lungs showed exudation into the alveolar lumen sufficiently marked to be considered the predominant lesion and were classed as stages of beginning red hepatisation.

DISCUSSION

The earlier studies on the production in the lower animals of lobar pneumonia with the pneumococcus were concerned at first with establishing the etiological rôle of this micro-organism.

The methods employed were direct injection of lung exudates into the thoracic cavity, trachea, peritoneum, and blood.

To Thalamon (3) is given the credit for first producing pulmonary lesions in rabbits by the intrathoracic method. Gameleia (4) carefully worked out the

relative resistance or susceptibility of the various lower animals to intraperitoneal injections of pneumococcus. He found that white mice and rabbits are most susceptible to pneumococcus infection while guinea pigs, dogs, sheep and man are increasingly more resistant. He produced pneumonia by intrathoracic inoculation in rabbits, dogs, and sheep. Gamaleia further showed that, since virulent strains would rapidly kill susceptible animals with an overwhelming septicemia without any pulmonary localisation, in order to produce pulmonary lesion in these animals an attenuated strain must be used. Because of this fact later investigators have attempted to determine what factors determine pulmonary localisation of the infection in susceptible animals. Wadsworth (5) expressed the belief that there exists a subtle equilibrium between the resistance of the animal on the one hand and the virulence of the organism on the other. He found that normal rabbits died of a fulminating septicemia without pulmonary lesions following intra-tracheal injections of highly virulent pneumococci. But on the other hand he could cause pneumonia to develop in normal animals by the use of less virulent cultures or by the use of virulent cultures in animals which had acquired a certain degree of immunity. In dogs, less susceptible animals, which were used by Lamar and Meltzer (6) and Wollstein and Meltzer (7), pneumonia was constantly induced after intra-bronchial insufflation of large amounts of culture. More recent work by Blake and Cecil (8) on monkeys has emphasised the bronchiogenic origin of the disease and these observers state that "lobar-pneumonia has been consistently produced in normal monkeys by the intra-tracheal injection of minute amounts of pneumococcus culture." Winternitz and Herschfelder (9), however, working on rabbits had previously found that it was necessary to introduce the organisms deep into the lower respiratory tract to produce pneumonia and Winternitz (10), considers that the lymphatic origin of the disease cannot be eliminated, in spite of the experiments quoted above, on the ground that by the method of intra-tracheal inoculation it is impossible not to infect the peribronchial tissue as the needle is being withdrawn.

It will be readily noted that all the "successful results" reported, without discriminating between those of doubtful and actual value, have been by intra-thoracic or intra-tracheal inoculation, there being no experimental proof of the haematogenous origin of the disease. But even the success of these methods has not been universally accepted, as many observers (Welch & Canfield (11), Krause and Pansini (12)) failed to confirm Gameleia's results, and succeeded only in producing lesions which they did not consider comparable to the typical lobar pneumonia as seen in man.

The present method, that of inhalation, is generally admitted to have failed, although some investigators believed that an occasional positive result was obtained. Selter (13) was unable to produce pneu-

monia in rabbits and guinea pigs exposed in a box to a spray of pneumococci which had been isolated from an epidemic of pneumonia in these animals. The inhalation method was employed in the present experiments because it is less artificial than the others and removes the objection of mechanical injury.

The points which must be considered in any discussion of the pathogenesis of pneumonia are: 1—the route by which the causative agent reaches the lung and 2—the site of initial invasion and spread of the infection in the pulmonary tissue.

Mode of Invasion

The infecting organism may reach the lungs by the blood stream, lymphatics, or directly from the respiratory tract.

The lymphatic route presupposes that entrance takes place in the bronchi or upper respiratory tract and that the organisms progress along the lymphatics to the lymph nodes at the root of the lung. The lymphatics become blocked by the inflammatory reaction set up by the organisms causing a back pressure and the infection is first noticed in the lung tissue. Or the organisms may pass through the lymph nodes into the thoracic duct and reach the lung by the pulmonary artery. In the mouse the lung is scant in lymphatics and we have seen no evidence in our sections of any such marked lymphatic involvement as this mode of infection would presuppose. It is certain that the lymph nodes themselves are not early affected.

The occurrence of pulmonary changes in the presence of negative blood cultures in the first 6 hours would at first sight appear to favor theory of direct primary invasion of the pulmonary tissue itself, particularly as the earliest lesions encountered have always been in the alveolar wall. But these pathological findings may be equally well explained on the ground that the infecting agent reaches the alveolar wall by the capillaries and is localised there until later when a secondary septicemia occurs.

The adherents of the hematogenous method of infection assume that the pneumococcus gains entrance at some point in the respiratory tract and is carried either directly into or through the lymphatics into the blood stream to the lungs where the microorganisms set up a

pneumonia. A selective filtration action on the part of the lungs is assumed to take place. No direct experimental evidence has yet been brought forward to prove this hypothesis. Attempts to test its validity are now in progress. The fact that pneumococci may be recovered from the liver, spleen and kidney of rabbits and guinea pigs following inhalation of pneumococci, though the blood culture is negative, indicates that a transient pneumococcus septicemia may occur. It has further been shown by daily blood cultures following spraying that a transient pneumococcus septicemia occurs frequently in rabbits. There probably are few organisms free at any one time in the circulating blood and furthermore these few bacteria may be within leucocytes, not multiplying in the blood. The occasional organisms which reach the blood are probably rapidly filtered out by the tissues and locally destroyed.

The failure to obtain positive blood cultures in the 2 mice which showed evidence of pulmonary lesions may be due to the circumstance that re-invasion of the blood stream from these foci of infection had not taken place after, possibly, transient primary septicemia. 2 of the 4 mice killed after 18 hours, however, with lungs showing lesions, had a generalised septicemia.

Nature of the Initial Lesion

In a proportion of partially immunised and intoxicated mice, infection after inhalation of virulent pneumococci is localised in the lungs. In these mice the initial lesion is an inflammation of the alveolar wall (extra or intra vascular). Blake and Cecil incline to the belief that the early lesion is in the interstitial tissue, while Permar (14) speaks of the disease as an exudative alveolitis throughout. Since cellular exudation into the alveoli occurs very early, the nature of the initial lesion can only be determined in mice killed soon after infection. But these early lesions are so minute that there is no gross pathological change except perhaps congestion, the significance of which is debatable in sacrificed animals. The only safe method is to cut serial sections of the whole lung in order to avoid missing any minute early lesions.

In the present study localisation of the pneumococcus infection was present in the lungs of 7 of 71 mice. Although this is too small a

number of animals from which to draw definite conclusions, the outcome agrees with that of previous experiments in which the observations were made on mice which had succumbed to the infection. The lymphatic spread from the original focus as observed in monkeys by Blake & Cecil certainly does not occur in the mouse. The infection in this species is progressive and is localized to the alveolar wall. In previous experiments pleurisy occurred when extensive lymphatic involvement was found and the animals died before a well defined localisation had time to develop. We believe that experimental pneumococcus pneumonia, at least in the mouse, begins in the alveolar wall, possibly within the capillaries.

SUMMARY

1. The serial section examination of 71 partially immune alcoholised mice which were killed at intervals following the inhalation of virulent pneumococci showed pulmonary localisation in 7 or 9%.

2. In the case of the mouse the initial lesion of pneumococcus pneumonia is in the alveolar wall and the exudate into the alveolar lumen occurs secondarily.

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STUDIES ON INFLAMMATION

II. A Measure of the Permeability of Capillaries in an Inflamed Area

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(Received for publication, November 12, 1929)

The accumulation of vital dyes in areas of inflammation has been demonstrated by several investigators. MacCurdy and Evans (1) pointed out that the normal brain and cord always remain free from dye injected intravenously but that areas of damage, such as softening or inflammation, become deeply stained. Bowman, Winternitz, and Evans (2) found that trypan blue injected intravenously stains tubercles in experimental tuberculosis. Subsequently Winternitz and Hirschfelder (3) demonstrated that this dye when injected in experimental lobar pneumonia stains the consolidated area of lung selectively: "The intravenous injection of trypan blue and trypan red gave rise to the usual diffuse staining as described by Bouffard, Goldman, etc., but in addition to this the diseased area of lung showed a much more intense staining than any of the other tissues, while the normal lung tissue was practically normal in color." Lewis (4) found that if the cornea of a rabbit is inoculated with a living culture of the tubercle bacillus, a progressive lesion results characterized by an intense congestion of the conjunctiva. If the animal receives an intravenous injection of trypan red 24 hours or more after such inoculation, the fluid in the anterior chamber of the inoculated eye always becomes colored. Precisely similar results were obtained when abrin was administered in the conjunctiva as an inflammatory irritant. A few years ago McClellan and Goodpasture (5) showed that trypan blue accumulates in lesions of herpetic encephalitis in the rabbit's brain, the injured areas presenting a striking color against the quite unstained healthy brain tissue. Siengalewicz (6) pointed out that general damage to the nervous tissue, such as poisoning with carbon monoxide or with salvarsan, is followed by marked staining of the damaged areas by trypan blue. Ramsdell (7) injected trypan blue into the veins of rabbits and guinea pigs previously treated with foreign serum and found that injection of the same serum into the skin of the ear immediately caused local infiltration of the dye into the adjacent tissue. She regarded the infiltration of the dye as an indicator of edematous changes resulting from toxic injury to the capillary endothelium.

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Okuneff (8) found that a thermal irritant favors the passage of vital stains from the blood stream into the area heated. Kusnetzowsky (9) also observed that the local application to the skin of an irritant such as heat or mustard oil causes an accumulation of trypan blue in the inflamed area when the dye has previously been injected into the blood stream. It has recently been demonstrated by one of us (10) that trypan blue injected into the circulating blood enters the site of inflammation rapidly and is fixed there, so that the tissues are deeply stained. Furthermore trypan blue injected directly into the site of inflammation in the subcutaneous tissue or in the peritoneal cavity is fixed in the inflamed area and fails to reach the regional lymphatic nodes. This work clearly showed that there is not merely a rapid accumulation of the dye from the blood stream into an inflamed area, but also that the dye is held in such an area and is unable to drain away through the regional lymphatics.

The object of the work reported here was an attempt to study quantitatively and directly the rate of change of concentration of trypan blue in the capillaries of an inflamed area. It was thought that such direct studies correlated with the preceding studies on the accumulation of dye from the circulating blood into an inflamed area might give some information concerning the change in capillary permeability with inflammation.

Method

An inflammatory reaction was produced in the peritoneal cavity of frogs (R. catesbiana and R. clamitans) (11) by the injection of about 2 cc. of either 5% aleuronat in Ringer solution or 4% turpentine in olive oil (12). Twelve to twenty-four hours after the injection of the irritant the brain of the frog was pithed, the spinal cord being kept intact in order to preserve a more adequate circulation (13). The frog was then placed on a frog board of the usual type and the abdominal wall was incised from the region of the pubis to the sternum. A loop of intestine was gently drawn out and the mesentery spread over a glass cover on the center of the board, which in turn was placed on the stage of the microscope. 1.5 cc. of aqueous 1% trypan blue was then injected directly into the ventricle. The dye stained the plasma of the mesenteric capillaries almost immediately. The circulation in most cases remained unimpaired. Gradually, over a period of about 10 minutes or less, most of the dye diffused out of the capillaries.

All these observations were duplicated in about the same number of normal animals as controls. Throughout the experiments particular attention was paid to the character of the peritoneal exudate, the appearance of the mesentery and the activity of the capillary circulation. When the circulation is in partial or complete stasis the capillaries may show variable changes in concentration of the dye, and these changes therefore, were studied only in capillaries in which the circula-

tion was active. Although prolonged exposure of the mesentery in a normal frog is sufficient injury to set up an inflammatory reaction, the duration of each experiment was so short that this factor, as judged by the scant migration of leucocytes in our control series, was almost negligible.

A graded series of standards of trypan blue ranging in concentration from 20 mgm. per 100 cc. of water to pure water was made up in glass capillary tubes measuring about 4 to 5 cm. long and about 1 mm. in diameter. These were glued on white cardboard so as to be conveniently handled by the observer. The determinations were made colorimetrically, the attempt being to match the dye in the plasma as seen through the low power objective with the dye in the set of standards. With only a little practice we found this method of estimating the concentration of dye in the plasma to be without difficulty. The observations were usually checked by two people and the mean taken. To avoid preconceptions, one investigator would often make readings while ignorant as to whether the mesentery under observation was inflamed or normal. Readings were made approximately every minute, the last not more than 7 minutes after the first since after that time the dye in the plasma was so dilute as to make accurate determination of the concentration questionable. Although the dye seen through the microscope in the capillaries was matched against standards in glass capillary tubes, nevertheless, since the same technique was used in estimating changes in concentration in both inflamed and normal areas, it is believed that these colorimetric measurements have yielded comparable and valuable data even though we cannot assume that they measure quantitatively the dye within the capillaries.

RESULTS

In Table I there are tabulated the results obtained for changes in concentration of trypan blue in the capillaries of the normal mesentery with disappearance of the dye. The results are expressed in units comparable to milligrams of dye per 100 cc. as determined by the standard scale, and the observations range over a period of seven minutes. The averages of the observed values are represented in Chart I.

If the concentration of dye is denoted by y and the time by x, and if log y for each concentration is plotted against x, a straight line is obtained. This indicates that the relation between concentration of dye in the capillaries and time follows an exponential curve of the "die-away" type: $y = be^{-ax}$, where b and a are constants, and e is the base of the natural system of logarithms equal to 2.71 . . . By the method of least squares the following equation is obtained:

$$y = 7.63\, e^{-.24x} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1)$$

TABLE I

Changes in Concentration of Trypan Blue in the Capillaries of the Normal Mesentery

Frog No.	Time in minutes						
	0	1	2	3	4	6	7
	Units comparable to milligrams per 100 cc.						
1	10	4	3	3	2.0	1.6	—
2	10	7	5	3	1.6	1.6	—
3	10	7	7	5	3.0	3.0	2.0
4	8.5	—	4	—	—	—	2.0
5	10	—	—	5	—	1.6	—
6	7	—	—	—	2.0	—	1.3
7	10	—	4	3	—	—	—
8	8.5	—	—	4	—	—	1.06
9	7	5	4	3	3.0	1.5	1.5
Average....	9.00	5.75	4.50	3.71	2.32	1.86	1.57

TABLE II

Changes in Concentration of Trypan Blue in the Capillaries of the Inflamed Mesentery

Irritant	Frog No.	Duration of inflammation	Time in minutes						
			0	1	2	3	5	6	7
		hrs.:mins.	Units comparable to milligrams per 100 cc.						
Aleuronat	10	12 : 30	10	4.0	2.8	1.6	1.3	—	1.2
	11	20 : 30	10	—	—	4.0	1.0	0.0	
	14	15 : 00	10	4.0	1.6	1.06	—	0.73	0.0
	16	18 : 00	10	2.0	—	0.37	—	0.0	
	18	17 : 30	10	—	1.6	0.0			
	19	23 : 00	7	3.0	2.0	1.0	0.7	0.7	0.0
	20	17 : 00	10	5.0	4.0	2.0	1.0	1.0	1.0
Turpentine	12	46	7.8	—	2.8	1.3	0.0		
	13	17 : 30	8.5	3.0	1.06	1.06	—	0.0	
	15	20 : 00	10	7.0	—	4.0	1.3	0.73	0.0
	17	18 : 00	10	—	—	4.0	—	1.3	0.0
Average.....			9.39	4.00	2.27	1.85	0.88	0.56	0.37

From this equation the calculated values of y are obtained:

Time (x) mins.	Concentration of dye (y) Units comparable to milligrams per 100 cc.	
	Observed	Calculated
0	9.00	7.63
1	5.75	6.00
2	4.50	4.72
3	3.71	3.71
4	2.32	2.92
5	—	2.30
6	1.86	1.81
7	1.57	1.42

These values are graphically represented in Chart I. It is seen that there is a fairly close agreement between the calculated and observed figures.

The results obtained for the changes during the first seven minutes in the concentration of trypan blue in the capillaries of the inflamed mesentery are tabulated in Table II. As with the normal values, plotting log y against x points to an exponential type of relationship between the concentration of dye in the plasma and the time (Chart I). The equation obtained by the method of least squares is:

$$y = 6.9\, e^{-.42x} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

The calculated and observed values of y, graphically shown in Chart I, are as follows:

Time (x) mins.	Concentration of dye (y) Units comparable to milligrams per 100 cc.	
	Observed	Calculated
0	9.39	6.90
1	4.00	4.53
2	2.27	2.98
3	1.85	1.96
4	—	1.29
5	0.88	0.85
6	0.56	0.56
7	0.37	0.37

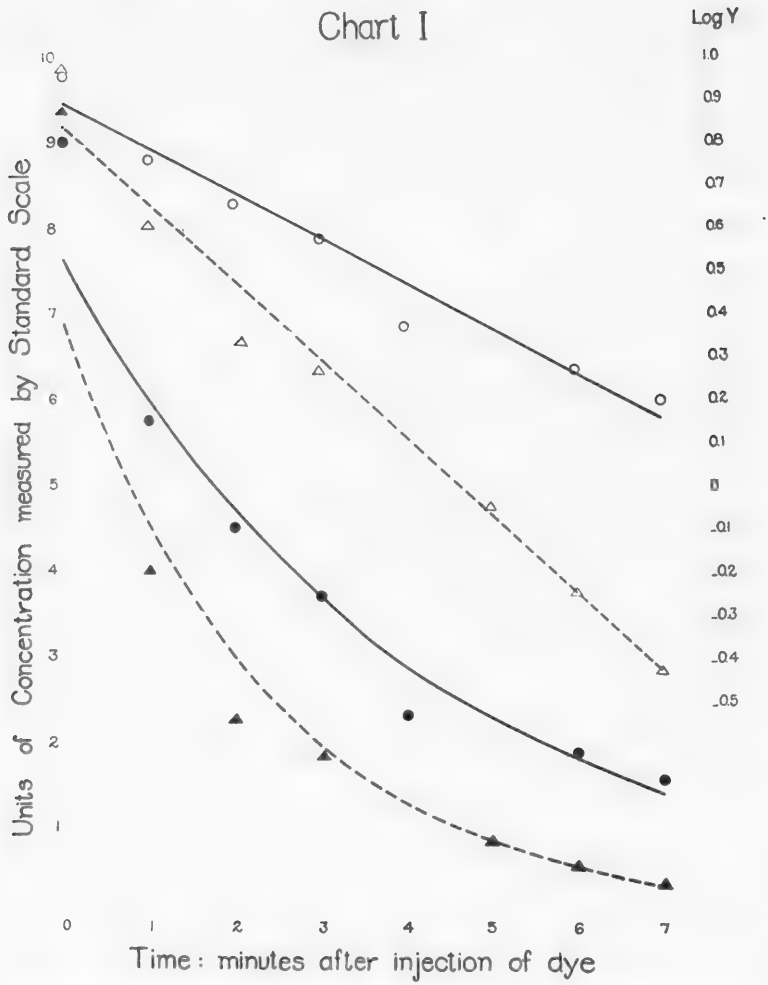

CHART I. The change in concentration of trypan blue in the capillaries of the mesentery. ● observed normal values. ▲ observed values, inflamed mesentery. ———— concentration of dye in capillaries of normal mesentery (calculated). ---------- concentration of dye in capillaries of inflamed mesentery (calculated). ○ logarithm of observed normal values. △ logarithm of observed values, inflamed mesentery.

Both equations (1) and (2) appear to be the nearest expressions that describe the observed facts. It is seen that in equation (1) a equals 0.24, whereas in equation (2) it equals 0.42. Since a is an index of the slope and consequently of the rate of change of concentration of the dye it is clear therefore that the rate of fall of concentration of trypan blue in the capillaries of an inflamed area is almost twice as rapid as that found in the capillaries of the normal mesentery.

A question now presents itself. Is the rate of change of concentration of the dye in the capillaries an indication of the rate of passage of the dye outward into the extra-capillary spaces? The dye obviously diffuses outward into the extra-capillary spaces, as can be seen by direct observation, and, of course, by the fact that the dye stains the cells of the extra-capillary spaces. This does not necessarily mean that the fall in concentration of dye within the capillaries is a measure of the amount of dye that passes through their walls, for it is conceivable that there may be other factors that change the concentration of dye within the capillaries in an inflamed area. However, in view of previous studies (10) in which it has been shown that trypan blue injected intravenously rapidly passes into an inflamed area, it is believed that the increased rate of fall of concentration is a measure of increased passage of the dye through the capillary wall.

DISCUSSION

The above observations show that the rate of fall of concentration of trypan blue in the capillaries after intraventricular injection of the dye is greater in the inflamed than in the normal mesentery. Landis (14) showed by micro-injection studies that the rate of passage of dye solution through the normal capillary wall appears to depend upon the level of capillary pressure, not upon capillary diameter. He demonstrated that the rate of filtration through the capillary wall was directly proportional to the difference between the capillary pressure and the osmotic pressure of the plasma colloids. He also emphasized (13) that the vitality of the capillary wall is of the utmost importance, since injury increases its permeability to proteins. At the same time he demonstrated that the increased filtration through the capillary wall with the use of urethane was due not to a stretched endothelium of the dilated capillary, as Krogh originally believed (15), but rather

to a direct injury of the endothelium by urethane accompanied by a high capillary pressure. Furthermore he showed that capillaries injured by alcohol and mercuric chloride appear to be permeable to the plasma colloids and approximately seven times more permeable to fluids than the normal capillary wall. In view of these facts it seems probable that the increased rate of fall in concentration of trypan blue in the capillaries of the inflamed mesentery might also be caused, as with urethane, alcohol, or mercuric chloride, by a direct toxic effect of the inflammatory irritant on the capillary endothelium, which would thus cause an increase of passage of the dye into the extra-capillary spaces. Such direct injury, causing a fall in the osmotic pressure of the plasma colloids resulting from increased capillary permeability, would adequately account for an increased rate of filtration of the dye in an inflamed area and therefore a greater rate of fall in the concentration of dye within the capillaries.

It is true that the factor of increased capillary pressure cannot be completely ruled out. The work of Hirschfelder (16) would however seem to rule out this factor as being of much significance in the increased filtration of dye in inflammation. This investigator showed that when adrenalin was used to reduce the filtration pressure between the capillaries and the extra-capillary spaces, preventing thus the development of edema, still the inflamed area was stained more deeply than the normal area when trypan blue was injected intravenously. For this reason it is believed that the increased rate of fall of concentration of dye in the capillaries of an inflamed area is primarily due to direct injury of the endothelium by the irritant causing rapid filtration of the dye with increased capillary permeability.

The exponential type of equation which seems to describe adequately the rate of change of concentration of trypan blue in the capillaries brings up an interesting point. This type of curve reaches the abscissa line only asymptotically. It would follow that in these experiments the plasma is never completely free of its dye content. This fact is in accord with the work of Grollman (17) who pointed out that a certain amount of such dyes in the circulation is bound by the plasma protein and therefore does not filter out of the capillaries.

We wish to express our thanks to Dr. Eugene L. Opie and to Dr. M. H. Jacobs for assistance in the preparation of this paper.

CONCLUSIONS

1. By means of a colorimetric method the concentration of trypan blue in capillaries can be estimated by direct observation and its changes followed as the dye passes out of the circulating blood stream.

2. The change in concentration of trypan blue in the capillaries of both the normal and the inflamed mesentery of frogs can be described by two separate exponential equations of the type: $y = be^{-ax}$.

3. From these equations it is found that the rate of fall of concentration following intraventricular injection of the dye is almost twice as great in the capillaries of the inflamed as in those of the normal mesentery. This difference is a measure of increased permeability with inflammation.

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THE IMMUNOLOGICAL SPECIFICITY OF CHEMICALLY ALTERED PROTEINS

Halogenated and Nitrated Proteins

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(Received for publication, October 9, 1929)

The immunological specificity of protein antigens has interesting aspects from the chemical viewpoint. Protein specificity appears to be closely associated with, and dependent on, chemical differences in the protein molecules. In most instances these differences are such that it is not possible to distinguish between similar proteins of different species by the usual methods of chemical investigation, but there are instances in which such a differentiation is possible by chemical or physical as well as by immunological tests. The differences which are responsible for serological specificity may not necessarily involve variations in the amount of certain special amino acids or even of free groups such as amino or carboxyl groups, but may be due merely to differences in the structural or spatial arrangement of certain groupings. A fundamental difference between antigenicity and specificity is indicated by the more recent work on complex antigens and haptens.

There are several methods applicable to the study of the relationship between immunological specificity and the chemical properties of antigens; (a) alterations in the chemical constitution of the protein can be effected by various chemical processes such as oxidation, iodination, nitration, esterification, etc. (Obermayer and Pick (1), Landsteiner (2)), (b) a comparison can be made of the immunological properties of closely related vegetable proteins (Wells and Osborne (3)) or animal proteins (cf. review by Wells (4) pp. 68–74), and (c) compounds of well-defined chemical nature can be attached to the protein molecule (Landsteiner (5)). Each of these methods has a special

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value in furnishing evidence about the nature of the groups which are concerned with specificity. The third method permits a study to be made of the effect produced by the introduction into the protein molecule of substances of related constitution and configuration, and many variations can be made in the constitution of the substituent group. The first method, the study of the specificity of chemically altered proteins, does not offer the same widespread opportunities since the methods of producing chemical changes in the protein molecule are rather limited; in addition there are few, if any, instances which cannot be objected to on the ground that the treatment to which the protein is subjected may produce other alterations besides that desired, for it is hardly possible to state with absolute certainty that the protein molecule can be attacked in such a way that only one significant change in the molecule occurs.

The first observations on the specificity of chemically altered proteins were made by Obermayer and Pick (1). The most significant changes were observed when serum was subjected to the action of iodine, nitric acid and nitrous acid and the so-called iodo-, nitro (xantho-) and diazo-proteins were obtained. These protein compounds still possessed antigenic properties but the chemical treatment had resulted in a loss of species specificity and the acquisition of a new specificity. Thus an iodoprotein from horse serum produced in a rabbit antibodies which reacted with iodized horse serum and with an iodoprotein derived from the serum of any species but not with native horse serum. Nitrated horse serum produced antibodies for any nitrated protein but not for horse serum or for iodized serum proteins. From these experiments the conclusion was reached by Obermayer and Pick (cf. also Pick (6)) that the species specificity of serum proteins is fundamentally related to the aromatic radicals present in the protein molecule and that any change in the aromatic nucleus destroys the original specificity and endows the molecule with a structural specificity characteristic for the new structure. Little evidence was available at that time however, that the action of iodine, nitrous acid or nitric acid was directed solely towards the aromatic amino acids, in particular the tyrosine molecules, and whilst these reagents are known to effect changes in the benzene ring, there may be other processes such as oxidation and hydrolysis which occur simultaneously. The methods of iodination used by Obermayer and Pick, iodination in hot acid solution (Hofmeister (7)) and iodination by the method of Blum and Vaubel (8) which involves treatment of the serum with iodine in the presence of sodium bicarbonate and heating at 50° for some time, will produce changes in the protein molecule other than simple iodination in the benzene ring. Also, in the preparation of nitrated and diazotized proteins, the treatment with acid may effect changes such as denaturation or hydrolysis.

Although in these three processes (iodination, nitration and diazotization) a distinct change occurs in the aromatic radicals of the protein, notably the tyrosine, the rest of the protein molecule is undoubtedly affected by the treatment.

One of the most interesting of these chemically altered proteins is the iodoprotein and several authors have obtained general confirmation of the observations made by Obermayer and Pick that treatment with iodine destroys, more or less completely, the original species specificity and occasions a new specificity.

Wells (9) obtained inconstant results with iodized serum in anaphylactic experiments with guinea pigs but with one preparation found that the iodized serum possessed the property of sensitizing guinea pigs to either the iodized serum or normal homologous serum. In general, however, no loss of species specificity occurred on iodination. Freund (10) carried out experiments similar to those of Obermayer and Pick and obtained confirmatory evidence that the specific complex of the protein is bound to the aromatic groupings of the molecule. Anaphylactic experiments have been carried out by various authors (11, 12, 13, and 14). Bauer and Murschhauser (15) described immunization experiments with iodized serum and iodized casein and conclude that only the labile proteins, such as serum proteins, lose their specificity on iodination and that the less labile proteins (cf. caseinogen) do not behave in this way.

The serological properties of nitrated proteins have recently been discussed by Ottensooser and Strauss (16).

There appears to be satisfactory evidence that the process of iodination leads to a more or less complete loss of species specificity and to the acquirement of a new specificity for iodinated proteins. It appeared desirable however, that more evidence should be available as to the nature of the changes involved in the iodination process before the loss of specificity is attributed solely to the introduction of iodine into the benzene ring of the tyrosine molecules present in the original protein. The view that the aromatic radicals largely determine the species specificity appeared to receive support from the fact that gelatin, which is deficient in aromatic amino acids, possesses no antigenic properties (Wells (9)). It is quite possible however, that the whole of the particular grouping or complex which is necessary for antigenicity may play little or no part in determining specificity, and indeed, the more recent observations on the haptens of pneumococci (Avery and Heidelberger) and the Forssman antigen (Landsteiner and Levene) indicate that the grouping which determines specificity may be entirely devoid of aromatic radicals. From the studies of the immunological properties of the iodoproteins and those of the nitrated proteins, it appeared probable, however, that the tyrosine groupings are concerned in some way with protein specificity, but the evidence upon which this view was based is not entirely convincing.

Several authors (Hofmeister (7), Hopkins (17), Blum and Vaubel (8) etc.) had observed that iodized proteins react negatively to Millon's test, a finding which appeared to justify the conclusion that substitution in the tyrosine nucleus occurs; but it was not until afterwards that Oswald isolated 3:5 diiodotyrosine from naturally occurring iodoproteins (18) and from artificially iodized proteins (19).

The methods of iodination used for the immunological experiments above mentioned—those of Hofmeister (7), and Blum and Vaubel (8)—may, as discussed above and as pointed out by Hofmeister, produce changes in the protein molecule in addition to simple substitution in the benzene ring of the tyrosine molecules. Iodization by the method of Blum and Vaubel leads, according to Blum and Strauss (20), to iodination in the tyrosine grouping, some oxidation processes and a change involving a destruction of one of the groups responsible for the biuret reaction. The latter authors also discuss the possibility of iodination in parts of the protein molecule which are unrelated to the tyrosine groupings. Very recently an investigation was made by Bauer and Strauss (20-a) on the chemical changes which occur in iodination and nitration of proteins. According to these authors nitration of proteins may involve a substitution in the tyrosine and tryptophane groups.

It seems desirable that a full investigation should be made of the serological properties of the halogenated and nitrated proteins with a view to gaining more information as to the rôle played by the aromatic groupings of the protein molecule in determining specificity. Consequently, halogenation or nitration has been effected by methods which have been modified in order to limit, as far as possible, the other changes such as hydrolysis and denaturation which readily occur in acid and alkaline solutions. For halogenation, bromine has been used in addition to iodine, and a study has been made of the serological properties of the brominated proteins.

EXPERIMENTAL

Preparation of Antigens

Iodinated Serum Proteins.

(a) This method was a modification of that of Blum and Strauss (21). Five hundred cubic centimeters of horse serum, 500 cc. of water and 500 cc. of 7% NH_4OH were mixed well and N/10 iodine solution added, with shaking, in quantities of about 50 cc. at each time. The iodine solution was added until free iodine was detectable by starch-iodide paper 5 minutes after the last addition of iodine, and then acetic acid was added to give maximum precipitation. The solution was centrifuged and the precipitate was dissolved in about 1500 cc. of distilled water by the aid of a little dilute Na_2CO_3 solution, the pH of the solution being kept below 8.5. The iodoprotein was reprecipitated twice more from a very faintly alkaline solution by acetic acid and then dissolved in 0.9% NaCl solution by the aid of a little dilute Na_2CO_3 solution. The reaction of the solution was adjusted to pH 7.5 and sufficient phenol to give 0.25% was added to that part of the solution required for injection. The horse serum iodoprotein, reprecipitated several times

from slightly alkaline solution by acetic acid and washed well with alcohol, contained 9.0% of iodine. Similar preparations were made with beef, chicken and rabbit serum. All the iodoproteins prepared by this method reacted negatively to Millon's test.

(b) *Iodination in Hot Acid Solution.* ((7.1) cf. Landsteiner and Prášek (22).) The brown product was dissolved as far as possible in dilute sodium carbonate solution and reprecipitated with dilute acetic acid. The product was then redissolved in 0.9% NaCl by the addition of a small amount of dilute Na_2CO_3. The reaction of the solution was adjusted to pH 7.5. This preparation and the corresponding antisera were used for the experiments recorded in table IX only.

Brominated Serum Proteins. A modification of the method of Hopkins and Pinkus (23) was found most satisfactory for the purpose. A mixture of 250 cc. of horse serum and 500 cc. of distilled water was treated with bromine water until free bromine could be detected after the solution had been well shaken and allowed to stand for a few minutes. One per cent sodium carbonate solution was then carefully added to give maximum precipitation and the solution centrifuged. The precipitate was dissolved in about 1 liter of water with the aid of a small amount of dilute Na_2CO_3, the pH being kept below 8.5 and the bromoproteins precipitated by the addition of a small amount of 10% acetic acid. This precipitate was dissolved in about 300 cc. of 0.9% NaCl by the addition of a little dilute Na_2CO_3, the reaction of the solution adjusted to pH 7.5, and sufficient phenol to give 0.25% was added to the solution used for animal injections. The brominated horse serum proteins, purified by several precipitations with acetic acid, washed with alcohol and dried, contained 4.7% of bromine. The brominated sera reacted negatively to Millon's test. Attempts were made to modify this process by brominating in approximately neutral solution by adding Na_2CO_3 or NaOH after the addition of bromine to the horse serum but the products obtained differed from that obtained by the method described above in their insolubility in neutral or faintly alkaline solution (pH 7.5 to 8.0).

Chlorinated Serum Proteins. A method analogous to the bromination method was used and a product obtained which had properties similar to those of the brominated proteins. A fairly considerable amount of chlorine was absorbed during the chlorination process but when the crude chloroproteins had been purified by several reprecipitations from slightly alkaline solutions with acetic acid and had been washed well with alcohol and then dried, they contained no combined chlorine. The results of the immunization experiments with the chlorinated proteins are, therefore, not included here since a comparison with the other halogenated proteins is not possible. The results showed that some slight loss of the original species specificity had occurred.

Nitrated Serum Proteins.

(a) With nitric acid ("xantho" proteins)—cf. Landsteiner and Prášek (22).

(b) With tetranitromethane ("nitro" proteins). One hundred cubic centimeters of serum, 100 cc. of pyridin, 200 cc. of distilled water and 30 cc. of tetranitromethane were mixed and shaken at intervals at room temperature. The mix-

ture assumed a reddish orange color which finally became orange brown, sometimes with the intermediate formation of a deep green colour. Further additions of pyridine were made to keep the solution neutral or faintly alkaline. The solution was centrifuged, after the addition in some cases of a small amount of dilute acetic acid to complete the precipitation, and the precipitate washed with about 200 cc. of acetone, centrifuged and washed twice more with about 200 cc. of acetone. The yellow precipitate was then suspended in water and dilute sodium carbonate added to render slightly alkaline. NaCl was added to give 1%, and the pH adjusted to 7.5. Sufficient phenol to give 0.25% in the solution was added to the suspension of nitrated horse serum used for the injections. The amount of protein in the filtered solutions was 0.5–1.5%.

Metaprotein. Twenty cubic centimeters of serum were mixed with 200 cc. of distilled water and 2 cc. of concentrated HCl (spec. grav. 1.18). The mixture was allowed to stand at 37°C. for 24 hours and the metaprotein precipitated by the addition of dilute sodium carbonate. The precipitate was washed with a little 0.9% NaCl solution and dissolved in water with the aid of a little dilute Na_2CO_3 or very dilute NaOH. The solution had to be kept slightly alkaline to prevent precipitation of the metaprotein. Thymol was added as a preservative.

Diazotized Serum Proteins. (cf. Landsteiner and Prášek (22).)

Immunization

Five rabbits received intravenous, or in some cases intraperitoneal, injections of the antigen at weekly intervals and the sera were tested 7 days after the third and, where necessary, the subsequent injections. The antigens used for injection were prepared from horse serum in every case.

Iodo-horse Protein. Five cubic centimeters of a solution containing 4.5% of iodoprotein were injected intravenously on the first occasion. Subsequent injections were made intraperitoneally since several fatalities occurred on the second intravenous injection. Two rabbits gave good antisera after three injections and two more after four injections.

Acid Iodinated Protein. (Hofmeister's preparation.) Two intravenous injections were made of 5 cc. of a solution containing 1.5% of iodoprotein, followed by intraperitoneal injections of 10–15 cc. of the solution at the subsequent injections. Four to six injections were necessary.

Brominated Protein. Intravenous injections were made of a solution containing 4% of bromoprotein. Two animals were bled after 3, one after 4 and one after 5 injections.

Nitrated Protein ("Xanthoprotein"). Intravenous injections were given of 5 cc. of a solution containing 2% of xanthoprotein. One animal bled after 3, one after 4, and one after 5 injections.

Nitrated Protein ("Nitroprotein") Intraperitoneal injections were made of 10 cc. of a suspension containing 2.5% of nitroprotein. Four animals bled 7 days after the third injection.

Precipitin Tests

For these tests the antigen solutions were diluted to 1:100, 1:500, 1:2500 and in certain cases 1:12500 in terms of a 5% stock solution, and 0.2 cc. used for each test. Two drops of the immune serum were added to each tube, the solutions shaken and readings made after 1 hour at room temperature (25 to 30°C.). The intensity of the reaction was indicated as follows:—(no reaction), f. tr. (faint trace), tr. (trace), ±, +, +±, ++, etc.

The first readings in the following tables are those recorded after 1 hour at room temperature and the second readings after 2 hours at room temperature and overnight in the ice box. In most of the tables the results of tests with two antisera only, and in certain instances with only one serum, are recorded. Other antisera gave similar results and in each case at least three antisera were tested.

The iodoprotein preparation and the iodoprotein antisera used for the following experiments, except those recorded in table IX, were those corresponding to the first method of iodination, namely, iodination in the presence of ammonium hydroxide.

The serological properties of the iodinated and brominated proteins were investigated by experiments the results of which are given in tables I, II, and III. From table I it will be seen that the iodoprotein antiserum reacts to the maximum extent with iodinated serum and somewhat less with brominated sera. The bromoprotein antisera react with brominated or iodinated proteins, to a slightly greater extent with the former. Iodination and bromination lead therefore to a more or less complete loss of the original species specificity (tables II and III), but the reaction between an iodo-horse antiserum and the homologous antigen is always more marked than that with any other iodinated proteins. The same holds true for the brominated proteins, and in all cases there seems to be a retention of some species characteristics. The effect is more pronounced when the readings are made after a shorter interval of time, e. g., 5 minutes after mixing.

The results recorded in table IV and V show that the proteins nitrated by the two methods used have similar serological properties and that nitration by either method leads to the acquirement of a new characteristic specificity.

Cross tests made between nitrated and halogenated proteins (table VI) indicate that some interaction occurs between certain halogenated proteins (in the main the brominated products) and antisera to nitrated proteins. In these cases there is no question of a species reaction since

TABLE I

Antigen	Antigen dilution	Iodo-horse antiserum				Bromo-horse antiserum			
		1635		1643		1822		1823	
Iodo-horse	1:100	++±	++±	+++	+++±	++±	++±	++±	+++
	1:500	+±	+±	+±	++±	+±	++	+±	++
	1:2500	±	+	±	+	+	+±	+	+
Iodo-beef	1:100	+±	++	+±	++±	++	++	++±	+++
	1:500	+±	+±	+	++	+±	++	+±	++
	1:2500	tr	±	tr	±	±	+	+	+
Iodo-chicken	1:100	+	+±	+	+	+±	+±	++	+++
	1:500	+±	+±	+	+±	+	+±	+	++
	1:2500	tr	±	tr	±	±	±	±	+
Iodo-rabbit	1:100	+±	++	+	+±	+±	+±	++±	+++
	1:500	+±	+±	+	+±	+±	++	+	++
	1:2500	±	+	±	+	±	+±	+	+
Bromo-horse	1:100	+±	++	+±	++±	+++	+++±	+++	+++
	1:500	+	+	±	+	+±	++	+±	++
	1:2500	tr	±	−	±	±	±	+	+
Bromo-beef	1:100	+±	++	+	++	++±	++	+++	+++
	1:500	±	+	±	+	+±	++	+±	++
	1:2500	tr	±	−	±	±	±	+	+
Bromo-chicken	1:100	+	+±	+	+	++	++	++	+++
	1:500	+	+	±	±	+	+	+	++
	1:2500	f. tr	tr	−	−	tr	±	±	+
Bromo-rabbit	1:100	+±	++	+	+±	++	+++	++	+++
	1:500	+	+	±	±	+±	++±	+	++
	1:2500	tr	±	−	−	±	+	±	+

the antigens tested were prepared from proteins different from that which gave rise to the antiserum. A similar cross reaction occurs between bromo-horse antibodies and nitrated proteins antigens (cf. table VII).

Table VIII gives the results of experiments with diazotized proteins and shows that diazoproteins and nitrated proteins are very closely related serologically. Thus diazo-beef and nitro-beef proteins, for

TABLE II

Antigen	Antigen dilution	Iodo-antiserum						Bromo-antiserum					
		1657		1635		1643		1822		1823		1821	
Horse serum	1:100	±	+	−	f.tr.	−	−	−	−	−	−	−	−
	1:500	±	+	−	f.tr.	tr.	tr.	−	−	−	−	−	−
	1:2500	tr.	±	−	f.tr.	f.tr.	tr.	−	−	−	−	−	−
	1:12500	−	−	−	−	−	−	−	−	−	−	−	−

TABLE III

Immune serum	Antigen dilution	Antigen					
		Iodo-horse		Bromo-horse		Horse serum	
Anti-horse No. 55	1:100	f.tr.	f.tr.	−	−	+++	+++±
	1:500	tr.	tr.	−	−	++	++
	1:2500	−	−	−	−	±	+
Anti-horse No. 1807	1:100	−	±	−	−	++	++±
	1:500	−	tr.	−	−	+	+±
	1:2500	−	−	−	−	±	+

example, react equally well with antisera to any type of nitrated protein investigated. Diazotized proteins, like nitrated proteins, also give some cross reactions with brominated proteins.

In table IX are given the results of experiments with the proteins iodinated in acid solution (Hofmeister's preparation). These antigens do not appear to be very active serologically but give antisera which react quite well with iodinated proteins prepared by the more gentle treatment (method (a)) or with brominated proteins. The antigens obtained by iodinization in acid solution gave, however, remarkably

weak reactions with antisera to iodoproteins prepared by the action of iodine in ammoniacal solution.

Since it has been shown (Landsteiner (24), Landsteiner and van der Scheer (25)) that the precipitin reaction of azo-proteins can be inhibited specifically by simple substances containing the specific reactive grouping, it was thought possible that similar tests would give some

TABLE IV

Immune serum	Antigen dilution	Antigen							
		Xantho-horse		Xantho-beef		Xantho-chicken		Xantho-rabbit	
Xantho-horse No. 1770	1:100	+	+±	±	±	tr.	±	tr.	±
	1:500	+±	++	+	+±	+	+±	+	+±
	1:2500	+	+	±	+	tr.	+	±	+
Xantho-horse No. 1766	1:100	+±	++	±	+	+	+	±	+
	1:500	+	++	+	+±	+	+±	+	+±
	1:2500	±	+	±	+	±	+	±	+
Nitro-horse No. 1879	1:100	++	++±	±	+	tr.	±	tr.	tr.
	1:500	++	++±	+	+±	+	+±	+	+±
	1:2500	+	+±	±	+	±	+	+	+
Nitro-horse No. 1881	1:100	++	++±	+	+±	+	+±	+	+±
	1:500	+±	++	+	+±	+	+	+	+
	1:2500	±	+	tr.	+	tr.	+	±	+
Anti-horse No. 1807	1:100	−	−	−	−	−	−	−	−
	1:500	−	−	−	−	−	−	−	−
	1:2500	−	−	−	−	−	−	−	−

The term "xanthoprotein" is used here to refer to the nitrated proteins obtained by treatment with nitric acid and "nitroprotein" for the products obtained by the tetranitromethane method.

indication as to the nature of the reacting groups of the halogenated proteins.

Inhibition tests with equimolecular quantities of various substances (table X) show that whereas tyrosine and several other amino acids have no inhibitory effect on the reaction between halogenated proteins and the corresponding antisera, 3:5 diiodo-, 3:5 dibromo- and 3:5 dichloro-tyrosine have a very marked inhibitory influence on the

TABLE V

Immune serum	Antigen dilution	Antigen									
		Nitro-horse		Nitro-beef		Nitro-chicken		Nitro-rabbit		Horse serum	
Xantho-horse No. 1770	1:100	+	+±	±	+	+	+	±	+	−	−
	1:500	+	+±	±	+	±	+	±	+	−	−
	1:2500	f. tr	±	−	±	f. tr.	±	f. tr	±	−	−
Xantho-horse No. 1764	1:100	+	+±	±	+	+	+±	±	+±	−	−
	1:500	+	+	±	+	+	+	±	+	−	−
	1:2500	tr	+	−	±	tr	+	±	+	−	−
Nitro-horse No. 1879	1:100	++±	+++±	+	++	+±	++	+	+±	+	++
	1:500	++	+++±	+±	++	+	++	+	++	±	+±
	1:2500	+	+±	±	+	±	+	±	+	±	+
Nitro-horse No. 1881	1:100	++±	+++±	+	++±	+	++	+	++	±	+
	1:500	++	+++±	+	+±	+	++	+	++	±	+
	1:2500	+	+±	±	+	±	+	±	+	f. tr	±
Anti-horse serum No. 1807	1:100	−	−	−	−	−	−	−	−	+±	++±
	1:500	−	−	−	−	−	−	−	−	+	+±
	1:2500	−	−	−	−	−	−	−	−	±	+

See note at the bottom of table IV for an explanation of the terms xanthoprotein and nitroprotein.

iodoprotein or bromoprotein precipitin reaction. It is interesting to note that the diiodo-compound is most active in this respect and the dichloro-compound least active. Stronger solutions of all three dihalo-

TABLE VI

Immune serum	Antigen dilution	Antigens							
		Iodinated serum				Brominated serum			
		Beef		Chicken		Beef		Chicken	
Xantho-horse No. 1770	1:100	−	−	−	−	±	+	tr	±
	1:500	−	−	−	−	±	±	tr	tr
	1:2500	−	−	−	−	−	tr	−	−
Xantho-horse No. 1766	1:100	−	±	−	tr	±	+	±	+
	1:500	−	tr	−	tr	±	±	tr	±
	1:2500	−	f.tr	−	f.tr	tr	tr	−	f.tr
Nitro-horse No. 1879	1:100	tr	±	−	tr	±	+	±	+
	1:500	tr	+	−	tr	tr	±	tr	±
	1:2500	f.tr	tr	−	−	f.tr	tr	−	f.tr
Nitro-horse No. 1883	1:100	−	tr	−	−	±	+	tr	±
	1:500	−	tr	−	−	±	±	f.tr	f.tr
	1:2500	−	−	−	−	−	tr	−	−

TABLE VII

Antigen	Antigen dilution	Antigens							
		Xantho-proteins				Nitrated proteins			
		Beef		Chicken		Beef		Chicken	
Iodo-horse No. 1635	1:100	−	−	−	−	−	−	−	−
	1:500	−	−	−	−	−	−	−	−
	1:2500	−	−	−	−	−	−	−	−
Bromo-horse No. 1823	1:100	−	−	−	−	tr	±	tr	±
	1:500	−	−	−	−	f.tr	±	tr	±
	1:2500	−	−	−	−	−	f.tr	−	f.tr
Anti-horse serum No. 1807	1:100	−	−	−	−	−	−	−	−
	1:500	−	−	−	−	−	−	−	−
	1:2500	−	−	−	−	−	−	−	−

genated tyrosine compounds inhibit completely or almost completely the reaction between iodoprotein antigen and iodoprotein antiserum, and that between the brominated protein antigen and bromo-protein

TABLE VIII

Antigen	Antigen dilution	Immune serum															
		Xantho-horse				Nitro-horse				Iodo-horse				Bromo-horse				Anti-horse serum	
		1770		1766		1879		1881		1635		1657		1822		1823		1807	
Diazo-horse	1:100	tr	+	+	+±	+±	++±	+±	++	−	f. tr	tr	±	−	±	tr	+	−	f. tr
	1:500	+	+±	+	++	+±	++±	++	++±	−	tr	±	+	−	+	f. tr	+	−	−
	1:2500	±	+	+	+±	+	+±	+	+±	−	f. tr	f. tr	tr	−	tr	−	±	−	−
Diazo-beef	1:100	tr	±	±	+±	tr	+	+	+±	−	f. tr	tr	±	−	tr	tr	±	−	−
	1:500	±	+	+	++	+	++	+	++	−	−	f. tr	−	−	±	f. tr	±	−	−
	1:2500	±	+	±	+	±	+±	+	+±	−	−	−	−	−	tr	−	tr	−	−
Diazo-chicken	1:100	tr	tr	+	+±	±	+±	+	+±	−	f. tr	−	−	−	−	tr	±	−	−
	1:500	+	+±	+	++	+	++	+	++	−	−	−	−	−	tr	f. tr	±	−	−
	1:2500	±	±	±	+	±	+	±	+	−	−	−	−	−	f. tr	−	tr	−	−
Diazo-rabbit	1:100	±	±	+	+±	±	+±	+	++	−	−	−	−	−	f. tr	±	+	−	−
	1:500	+	+±	+	++	+	++	+	++	−	−	−	−	−	tr	±	±	−	−
	1:2500	±	+	±	+	±	+±	+	+	−	−	−	−	−	tr	f. tr	tr	−	−
Xantho-horse	1:100	+	+	+±	++	+±	++±	++	++±	tr	tr	f. tr	tr	f. tr	+	tr	+	−	−
	1:500	+	+±	+±	+±	+±	++±	++	++±	−	−	−	±	tr	+	f. tr	+	−	−
	1:2500	±	+	+	+	+	+±	+	+±	−	−	−	f. tr	−	tr	−	±	−	−
Xantho-beef	1:100	tr	±	±	+	tr	+	tr	+	−	−	−	f. tr	−	f. tr	f. tr	f. tr	−	−
	1:500	+	+	+	++	+	++	+±	++	−	−	−	−	−	−	−	−	−	−
	1:2500	±	+	±	+	±	+±	+	+±	−	−	−	−	−	−	−	−	−	−

TABLE IX

Immune serum	Antigen dilution	Antigen													
		Iodoproteins (Hofmeister's preparation)								Iodoproteins				Bromoprotein		Horse serum	
		Horse		Beef		Chicken		Rabbit		Horse		Beef		Beef			
Anti-iodoprotein (Hofmeister's preparation) No. 1771	1:100	±	±	tr	±	−	±	−	f. tr	++	+++	+±	++±	+	++	−	−
	1:500	±	+	f. tr	±	−	tr	−	±	+±	+++	+±	++±	+±	++	−	−
	1:2500	−	±	−	f. tr	−	−	−	tr	+	+±	+	+±	±	+	−	−
Anti-iodoprotein (Hofmeister's preparation) No. 1772	1:100	±	±	f. tr	tr	−	f. tr	−	−	++	+++	±	+	±	+	−	−
	1:500	tr	+	−	+	−	tr	−	tr	+±	+++	+±	++±	+	+±	−	−
	1:2500	−	±	−	f. tr	−	−	−	f. tr	+	+±	+	+±	tr	±	−	−
Anti-iodo-horse No. 1643	1:100	−	tr	−	tr	−	−	−	−	++	+++	+	++				
	1:500	−	f. tr	−	f. tr	−	−	−	−	++	++±	+±	++				
	1:2500	−	−	−	−	−	−	−	−	+	+±	±	+				

TABLE X

Inhibition Tests. 0.2 cc. of a solution of the substance tested for inhibiting action (containing 0.5 millimol per 100 cc. of 0.9% NaCl in each case) + 0.05 cc. of the antigen solution + 2 drops of immune serum. The strength of the antigen solution for the first four horizontal columns was 1:100 and for the others 1:20, of a 5% stock solution.

Antigen	Immune serum	Control		Substances tested for inhibitory action																	
				Tyrosine		3.5 diiodo-tyrosine		3.5 dibromo-tyrosine		3.5 dichloro-tyrosine		Alanin		Phenylalanin		Histidin		Phenol		o-iodophenol		Potassium iodide	
Horse serum	Horse serum No. 1807	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±
Xantho-chicken	Xantho-horse No. 1770	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±
Iodo-chicken	Iodo-horse No. 1635	+±	++	+±	++	−	−	tr	±	±	+±	+±	++	+±	++	+±	++	+±	++	+±	++	+±	++
Bromo-chicken	Bromo-horse No. 1822	+	+±	+	+±	−	tr	f. tr	tr	±	±	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±
Bromo-chicken	Iodo-horse No. 1635	+	+±	+	+±	−	−	tr	tr	±	+	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±
Iodo-chicken	Bromo-horse No. 1822	+	+±	+	+±	−	−	−	f. tr	f. tr	tr	+	+±	+	+±	+	+±	+	+±	+	+±	+	+±

antiserum or the cross reactions between iodoproteins and bromoproteins. None of these inhibiting substances have any influence on the reaction between nitrated proteins and the corresponding antisera or on the reaction between horse serum antiserum and the homologous antigen, indicating that the inhibiting effect of 3:5 dihalogenated tyrosine on the iodoprotein and bromoprotein precipitin reactions is specific.

All the antisera were tested with metaprotein antigens (beef and chicken) but in no case was any positive reaction noted.

DISCUSSION

The results of the experiments with halogenated and nitrated proteins undoubtedly offer support for the view that the aromatic radicals of the protein molecule may be concerned with the determination of specificity; but there are changes in the protein molecule which do not appear to be concerned with the aromatic nuclei but which lead to a loss of species specificity. Such an effect is produced by esterification with acid alcohol, acetylation, or methylation (Landsteiner and Prášek (26), cf. also (27, 28)).

Whilst the methods previously used were rather drastic in nature, the methods of halogenation employed here were such that hydrolytic and oxidative changes were reduced as far as possible.

Iodination and bromination result in a more or less complete loss of the original species specificity and a new specificity characteristic of halogenated proteins is produced. The injection of iodinated horse serum into a rabbit gives rise to the formation of antibodies which react with iodinated heterologous sera or with the brominated sera. Similar antibodies are produced by the injection of brominated serum and in these antigens there appears to be some common active grouping which is responsible for the new specificity. This new grouping seems to be bound up with the tyrosine molecules and for several reasons it appears probable that the serological properties of the halogenated protein antigens are due to the disubstitution of halogen in the 3:5 position of the tyrosine molecules. Ample evidence exists for the view that iodoproteins contain the 3:5 diiodotyrosine structure (Oswald (18, 19), Blum and Vaubel (8)), but in addition the possibility that substitution occurs in other places, (e. g. in the histidine groups), must not

be overlooked. According to Blum and Strauss (21), however, gentle iodination in the presence of ammonium hydroxide does not result in any appreciable attachment of iodine to the histidine nitrogen atoms.

Iodination or bromination leads to a loss of the power to give a positive Millon's reaction and this loss is attributed to disubstitution of halogen in the tyrosine ring in the ortho positions relative to the hydroxyl group (Blum and Vaubel (8)). This disappearance of a positive Millon's reaction runs parallel in the experiments described here, with the loss of the original species specificity, and incomplete iodination results in the formation of a product which still gives Millon's reaction and retains some of the species specificity. More convincing proof of the view that the new specificity of halogenated proteins is dependent upon the presence in the protein of 3:5 dihalogenated tyrosine groupings is derived however from the inhibition tests, since 3:5 diiodotyrosine has a marked inhibitory effect on the reaction between iodoproteins and the iodoprotein antiserum whereas tyrosine and other amino acids have none. Thus the iodoprotein antibodies have some active grouping which has a special affinity for the 3:5 diiodotyrosine grouping and indeed for any 3:5 dihalogenated tyrosine structure, since the corresponding bromo- and chloro-derivatives of tyrosine have a similar combining capacity for iodoprotein, or bromoprotein antibodies. These inhibitory reactions appear to be specific, and from other evidence (Landsteiner (24)) it seems possible that reactions of this type will be useful for the determination of the structure or configuration of chemically altered antigens.

The results of the experiments with nitrated (or xantho) proteins comfirm the findings of Obermayer and Pick (1), that after nitration with concentrated nitric acid there is a more or less complete loss of species specificity with the production of a new specificity characteristic for nitrated proteins. This method of nitration undoubtedly results in fairly drastic hydrolytic, and possibly oxidative, changes, and hence it was considered desirable that some other method of nitration of proteins, preferably one in which strong acids are not used, should be devised. The new method adopted, a method based on that of Schmidt and Fischer (29) and involving nitration with tetranitromethane in neutral of slightly alkaline solution, yields nitroproteins which resemble, in general chemical properties and immunological

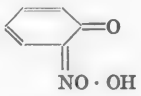
reactions, those obtained by the nitric acid method. Thus the antiserum to nitrated horse serum (tetranitromethane method) reacts with any nitrated serum or with the xanthoproteins (nitric acid method) from any serum, and the nitrated proteins react with the xanthoprotein antiserum.

A reinvestigation of some of the immumological properties of diazotised proteins has confirmed the observations of Landsteiner and Prášek (22) rather than those of Obermayer and Pick (1). The latter authors imply that xanthoprotein and diazoprotein are serologically well differentiated on account of the different nature of the substituents, and they conclude that substitution of a diazo group, presumably in the benzene ring, leads to a new structural specificity. Landsteiner and Prášek, however, found that the xanthoproteins and diazotised proteins show close serological relationship; thus the diazo antigens and the xantho antigens reacted almost equally well with diazotised protein antiserum or with xanthoprotein antiserum. In the experiments described here, diazotised serum proteins react like the xanthoproteins or the nitrated proteins and can hardly be differentiated in precipitation tests with xanthoprotein and nitrated protein immune sera. These facts seem to indicate that the diazotized protein and the nitrated protein have constitutions which are closely related.

Diazotization of proteins by the action of nitrous acid has proved difficult to account for chemically, since, according to all the evidence available, proteins contain no aromatic amino groups. Richard (30), who first described the reaction, reached the conclusion that proteins must contain aromatic amino groups, but another interpretation was advanced by Landsteiner (31) who showed that treatment of aromatic oxyacids, such as salicylic acid or tyrosine, with nitrous acid leads to the formation of diazo compounds (cf. also Weselsky (32), Morel and Sisley (33)). One may conclude that a similar process occurs when proteins are treated with nitrous acid, and that a diazo group is introduced into the tyrosine nucleus, probably in the ortho position to the hydroxyl group.

It is rather remarkable that diazotized salicylic acid and the diazotized proteins have a distinct yellow colour, comparable with that of the nitrated proteins. The marked yellow colour of the ortho-

nitrophenols has been attributed by Armstrong (34) to a quinoid structure (cf. also Hantzsch (35)).



This explanation might reasonably be extended to the case of the nitrated proteins and it is not improbable that a similar structure exists in the diazotized phenolic compounds or diazotized proteins. Such a change in the aromatic nucleus of the tyrosine molecule which leads to the formation of a quinoid structure might account for the serological alteration effected by nitration or diazotization of proteins. This view would offer a reasonable explanation for the close serological similarity between nitrated and diazotized proteins. At any rate it appears that a considerable difference in the nature of the substituent grouping has, in this case, no influence on the serological specificity.

The results of the work of Obermayer and Pick are generally accepted as a proof that the serological differences observed by the authors are merely due to chemical differences in the radicals introduced into the protein molecule. In the present investigation, however, an intense cross reaction between nitrated proteins and diazotized proteins and a strong interaction between iodo- and bromoproteins, were observed. Apart from slight cross reactions there was a sharp serological distinction between the nitrated and diazotized protein on the one hand and the halogenated protein on the other but this difference is not necessarily dependent upon the nature of the substituents only. It may depend on the place of substitution and on the number of entering groups which is probably different in halogenated and nitrated proteins (cf. 19, 36, 20-a).

It is not possible from the evidence available at the present time,—and to acquire more will be difficult,—to decide how far the introduction of different radicals into the same position of the aromatic nucleus would lead to serological differentiation. Thus it would be of interest to know whether the mere replacement of halogen atoms of halogenated proteins by, for example, hydroxyl, nitro, or methyl groups or by more complex radicals, would lead to the formation of antigens which would no longer react with antibodies to the halogenated pro-

tein. From other evidence, especially in regard to the azo-proteins (Landsteiner and van der Scheer (37)) it has been found that various substituents situated in the same position in the benzene ring do not necessarily occasion pronounced differences in serological behaviour.

In all the experiments with halogenated and nitrated proteins it has been observed that the antisera to a chemically altered horse serum react to the maximum extent with the homologus antigen. These differences, although distinct, are not so pronounced as some of those previously noted in the case of the xanthoproteins by Landsteiner and Prášek (22). These observations tend to indicate that the species characteristics are not entirely destroyed by nitration or halogenation, although the power to react with an immune serum to the native homologous serum may be entirely or practically entirely lost.

One possible explanation of these facts would involve the assumption that more than one group in the protein molecule is responsible for the species characteristics and that chemical treatment such as halogenation or nitration does not change or destroy the whole of these groupings. It is also possible that several active groupings of the antigen molecule combine with a similar number of groupings in the corresponding antibody and thus an antigen would give rise to the formation of an antibody which has its combining groups orientated in a manner similar to the orientation of the specificity groupings of the antigen. Thus the maximum reaction will be obtained only when the spatial distribution of the combining group or groups of the antigen and antibody are closely related, i.e., when the antiserum reacts with the homologous antigen.

SUMMARY

The serological properties of iodoproteins prepared by a method which involves less drastic treatment of the protein than the methods previously used for this purpose confirm the findings of Obermayer and Pick (1) and later authors, that iodination of proteins results in a more or less complete loss of species specificity and that a new specificity characteristic for iodoproteins is produced.

A serological investigation of brominated proteins has been made for the first time. These preparations are only slightly different from iodized proteins in their serological properties.

Evidence is submitted which indicates that the radical in iodoproteins which is responsible for the specificity is not iodine but the 3:5 dihalogenated tyrosine grouping. Thus marked inhibition of the iodoprotein (or bromoprotein) precipitin reactions is effected by 3:5 dihalogenated tyrosine, not by iodophenol or potassium iodide.

A reinvestigation has been made of the serological properties of nitrated and diazotized proteins. Proteins nitrated by nitric acid, or by a method which does not appear to have been used for proteins hitherto, namely nitration with tetranitromethane in neutral or slightly alkaline solution, acquire a new common serological specificity. The nitrated proteins and diazotized proteins show, in confirmation of the results of Landsteiner and Prášek (22) and in contrast to the findings of Obermayer and Pick, very little difference in their reactions. Thus diazotized proteins and proteins nitrated by either of the two methods above mentioned react equally well with any nitroprotein antiserum. This interaction exists in spite of the difference in the substituents, either because the substitution with the nitro- or diazo-group occurs in the same position in the aromatic nucleus, possibly in the ortho position to the hydroxyl group, or because of some other structural similarity. In the last connection it is suggested that both compounds may have a quinoid structure as has been assumed for ortho-nitrophenols.

Whilst this assumption could account for the marked serological difference of nitrated and halogenated proteins it should also be mentioned that iodination (and bromination) lead to a disubstitution of halogen in the two ortho positions relative to the hydroxyl group of the tyrosine whereas nitration of proteins probably results in the formation of mononitrotyrosine and substitution in the tryptophane group as well (19, 36, 20-a). It is probably impossible therefore, to draw a strict analogy between nitration (or diazotization) and halogenation of proteins since a comparison of their immunological properties is not exactly a comparison of the effect of substituting a different group in the same position. Accordingly it would appear that as yet no definite conclusions can be drawn as to the serological effect of differences in the chemical nature of various substituents in the aromatic nucleus although some influence is likely for general reasons.

All of the chemically altered proteins still retain a small amount of

the original species specificity, and the antisera always react to a slightly greater extent with the homologous antigen than with similarly treated antigens prepared from heterologous sera. This difference occurs even when the possibility of some unaltered protein being present in the antigen can be practically excluded.

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A CONCENTRATING DIALYZER

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(Received for publication, November 8, 1929.)

A device has already been described[1] for rapid and efficient dialysis of fluids placed in collodion bags. This device has been improved in several ways and may now be used not only to remove salts, sugars and other small molecules but also to concentrate simultaneously the remaining fluid to a small volume while keeping it at a low temperature and sterile. The improved dialyzer will be described below. It will concentrate a liter of an aqueous solution (such as whey or urine) to a few cubic centimeters (or complete dryness if desired) in 3 to 4 hours and, at the same time, remove the dialyzable materials, such as salts, sugars, etc. The apparatus is not intended to supplant devices for electrodialysis such as those designed by Bronfenbrenner and others, but to be used where electrodialysis is not desirable or not necessary.

Description of the Dialyzer

In the new dialyzer the fluid is contained in collodion bags at atmospheric pressure while cold water is passed around the bags under negative pressure, and the apparatus is kept rocking. The rocking accelerates the dialysis by agitating the fluid. It also distributes the fluid over the whole surface of the membrane. In this way the outward passage of water is independent of the volume of fluid almost to the end.

In Fig. 1 will be seen a perspective view of the dialyzer connected up for use. It consists of a 32 by 7 inch rocking board (with 2 by 7 inch pieces on the ends) which is supported above a 17 by 34 inch baseboard by 9½ by 2½ inch supports. Two heavy screws, 7 inches above the baseboard, serve as a pivot for the rocking board while a windshield scraper keeps it in motion.

Fifteen pyrex glass cells are fastened on the rocking board. In these cells are

[1] Kunitz, M., and Simms, H. S., *J. Gen. Physiol.*, 1928, **11**, 641.

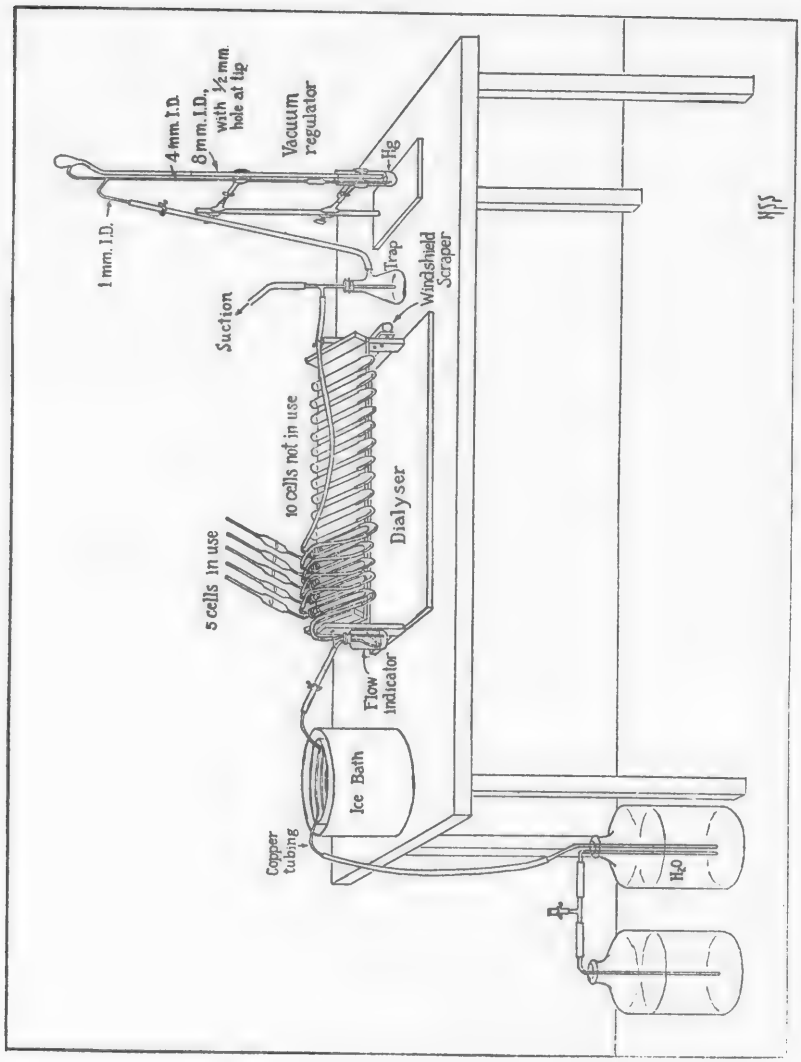

FIG. 1. Dialyzer in use

placed the collodion bags filled with the fluid to be dialyzed, each bag being connected with a pyrex reservoir bulb, as shown. Each bag holds 40 to 45 cc. of fluid, while 30 cc. more may be placed in each bulb. The details of the cells are shown more clearly in Fig. 2.

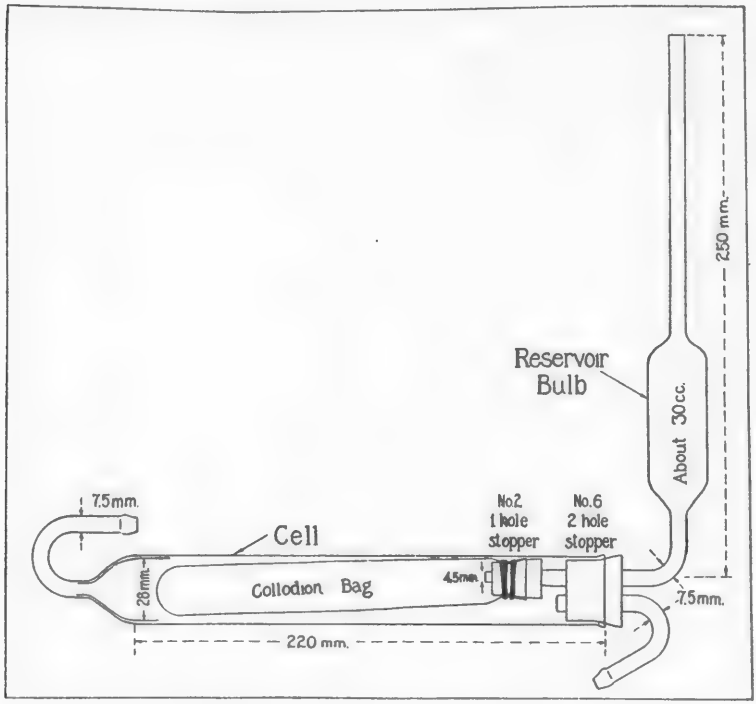

FIG. 2. Details of a cell and reservoir

The water (or salt solution) used for the "outside fluid" in dialyzing is placed in bottles on the floor and is sucked up, through an ice bath, into the apparatus where it passes first through the flow indicator and then successively through the 15 cells. The water then passes to a vacuum regulator and to a water suction pump. The vacuum regulator allows air to enter the system when the suction is too high. The ice bath keeps the dialyzed fluid below 10°C. For better cooling towels may be placed over the cells.

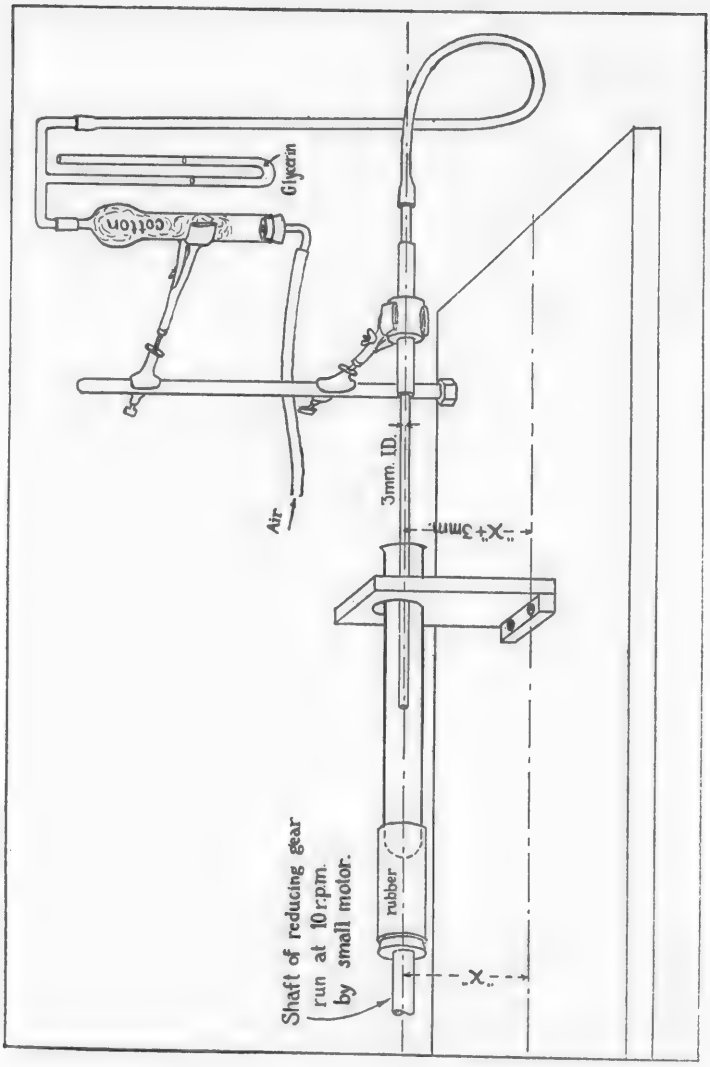

FIG. 3. Portion of machine for making collodion membranes[2]

Making the Collodion Bags

The method of making and testing the collodion bags is so important to the proper functioning of the apparatus that the technique is described in detail.

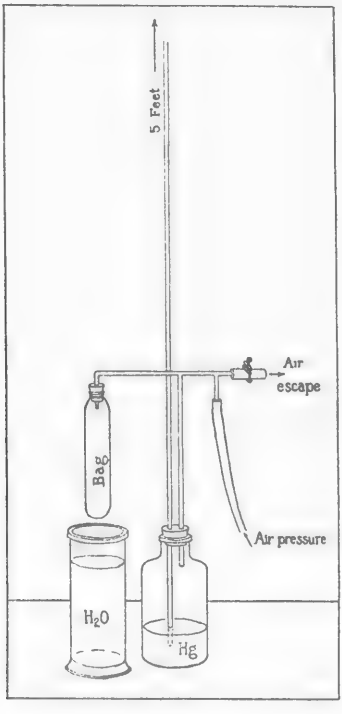

FIG. 4. *Bag Tester.* The air escape is gradually closed to increase the pressure in the bag. The cylinder of water is raised to immerse the bag.

The collodion bags should stand a test pressure of 70 cm. of mercury without leaking or breaking, and be so uniformly permeable that when filled with water and subjected to a 30 cm. pressure, the outside will immediately be completely covered with beads of water which has been forced through.

The membranes are made in pyrex test tubes 175 mm. long and *19 mm. inside diameter* (each tube should be measured). 10 cc. of Merck's collodion is placed in a tube and the tube is turned three times by hand to wet the whole surface. It is

then placed in a machine (see Fig. 3) which rotates it in nearly a horizontal position 15 times a minute for 10 minutes.[2] The angle of the test tubes in the machine is such that the mouth is ½ inch higher than the closed end.

The rotation is then continued 10 minutes longer while air is blown through a glass tube inserted about half the length of the test tube. The pressure and volume of air are so regulated that there is a pressure of about 1 cm. of *glycerine* at a point beyond which the air must pass through 36 inches of rubber and glass tubing with a 3 mm. inside diameter. The blowing tube should *not* be constricted at the end, to form a nozzle; on the contrary it is better to flare it slightly, but this is not necessary.

The tubes containing the membranes, thus made, are then removed, filled with water and allowed to stand 15 minutes before the bags are taken out. Each bag is then trimmed, placed tight on a No. 2 one-hole rubber stopper, and fastened with a No. 8 rubber band, which is wound around it about four times.

The bags are next tested with the device shown in Fig. 4. It is connected up with an air pressure system and the screw-lamp on the escape tube is closed until the desired pressure is obtained in the bag, as indicated by the mercury manometer. The pressure should be increased very slowly. When it is seen that the bag does not break (at say 60 cm. of mercury) a cylinder of water is brought up around the bag until the latter is completely immersed. Small holes will be shown by a stream of bubbles.

The bags are then filled with 60 per cent alcohol and stoppered with pieces of glass rod, tapered at one end and flattened like a nail head at the other. If the bags are to be used the same day they are immersed in distilled water until required. If they are not to be used until the next day they may be immersed in 60 per cent alcohol overnight and placed in water the next morning. For best results they should be used within 24 hours but they can be kept for two or three days if necessary.

Use of the Dialyzer

The bags are emptied by removing the plugs and repeatedly inverting them, to allow air to enter. Squeezing should be avoided. A second rubber band is then placed on each bag and the fluid to be dialyzed may be introduced with a pipette stuck into the hole of the stopper. Air is removed a little at a time by sucking on the pipette and then allowing the fluid to flow in. When a large number of bags

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[2] The machine in use can make two membranes at a time, one on each end of the shaft of a reducing gear. This is adapted from a machine made by J. H. Northrop and M. Kunitz (*J. Gen. Physiol.*, 1925–26, **9**, 315) in which one membrane was made at a time. Dr. Anson has further improved the apparatus so that it makes five membranes at once. His five tubes are parallel to each other and are connected by gears.

are to be filled it is easier to siphon the fluid into the bags using a glass tip of about 2 mm. outside diameter pushed far enough through the stopper to allow the escape of air.

The Pyrex reservoir bulbs are sterilized in an autoclave, a plug of cotton having been placed in the upper end. They are inserted in two-hole, No. 6 rubber stoppers and then filled like a pipette after inserting the tip in a short piece of rubber tubing attached to a glass tube projecting into a flask of the fluid.

After both a bag and a bulb are filled (preferably by different people) the two are joined and inserted in a cell of the dialyzer which contains a little water. When all the cells are thus filled the rubber connections are made and the suction is turned on to slightly more than the desired vacuum. Adjustment is made by means of the regulator to a negative pressure of about 30 to 40 cm. of mercury. A pinch-cock between the water bath and flow indicator regulates the flow of water to about 8 cc. per minute per bag. In the flow indicator the water squirts upward at an angle and gives an approximate idea of the rate of flow.

When the cells are full of water, and everything is adjusted, the windshield scraper is turned on at about 10 cycles a minute, rocking from about 45° forward to 45° backward. This is continued until the fluid inside the bags is sufficiently concentrated.

The apparatus with 15 cells will hold about 650 cc. of fluid if only the bags are filled and should concentrate this to a thick syrup in 1 to 2 hours. If the reservoirs are also filled over a liter of fluid may be handled at once and require 3 to 4 hours. Additional fluid may be placed in the reservoirs during operation. No aqueous solution should require over 6 hours unless it contains material which clogs the collodion membranes.

Concentration may easily be continued to complete dryness but it is best to stop before the removal of fluid from the bags becomes too difficult. When it is desired to remove one or more bags (or if a bag should break) pinch-clamps are placed on the water tubing on both ends of the dialyzer and the desired changes are made. The concentrated fluid may be removed by inserting the tip of a pipette in the stopper of each bag and sucking until the bag is collapsed. With the technique used the fluid may be kept sterile throughout the dialysis.

SUMMARY

An apparatus is described for dialyzing aqueous solutions and simultaneously concentrating them to a thick syrup, or even to dryness.

The fluid is placed in collodion bags with reservoir bulbs open to atmospheric pressure. The bags are surrounded by flowing cold water, under negative pressure, and are subjected of a rocking motion. Over a liter of fluid can be concentrated to a few cubic centimeters in 3 to 4 hours. It is kept cold and sterile during the process.

THE BACTERIAL GROWTH INHIBITOR (LACTENIN) OF MILK

I. The Preparation in Concentrated Form

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(Received for publication, November 8, 1929)

A substance capable of inhibiting the growth of certain bacteria is known to exist in cow's milk. This substance was shown, in a previous paper,[1] to prevent multiplication of scarlet fever streptococci until all eventually die. The mastitis streptococcus, however, is restrained for only a few hours, then growth suddenly begins and continues with extreme rapidity. This phenomenon was attributed to the development of a type resistant to the inhibitor since in the first place the inhibitory substance was not destroyed by this action, and in the second place the resistant streptococci are not restrained when transferred to fresh samples of milk.

In this paper the above described substance will be called *lactenin*;[2] and the potency of a solution or material in this substance will be referred to as its *lactenic* activity.

The lactenic activity of whole milk is known not to be decreased by the removal of the fat or by the coagulation of casein with rennet. Whey contains about 6 per cent solids, most of which are sugar and salts, while 0.4–0.5 per cent is protein. With this material as a starting point we have attempted to obtain as concentrated and pure a preparation as possible.

EXPERIMENTAL

In the following experiments milk was drawn directly from cleansed udders into sterile bottles and at once chilled. It was centrifuged and thus largely freed

[1] Jones, F. S., and Simms, H. S., *J. Exp. Med.*, 1929, **50**, 279.

[2] From the Latin: *lac*, milk; and *teneo*, hold.

from fat. The skimmed milk was always heated at 58°C. for 20 minutes and sufficient rennet solution added to insure coagulation. The rennet-milk mixtures were kept at 38°C. until about half the original volume could be withdrawn as whey. Later, when larger quantities were needed, the milk was pasteurized in centrifuge bottles, and after rennet coagulation, centrifuged at high speed.

Two methods were employed for test purposes. When the substances to be tested were regarded as sufficiently nutritive for bacterial growth to occur, they were merely inoculated with culture and incubated, and portions withdrawn and plated at intervals.

A second but simpler method for activity tests consisted in adding the test material to Petri dishes containing 0.5 cc. of defibrinated horse blood and pouring plates with melted agar inoculated with 16 hour broth cultures of the scarlet fever streptococcus. After 48 hours incubation the longest diameters of the hemolytic zones about several deep colonies were measured. An ocular micrometer and a mechanical stage micrometer were used with a 32 mm. objective.[3] It will be shown that such measurements offer a method of judging inhibitory activity. Data of this nature are given in Tables III A, IV A, and IV B. In each case a measurement indicates an average size of five hemolytic zones. These did not differ more than 0.1 mm. from the average. If the control has a size M_o and another measured zone has a size M, then $100\ M/M_o$ is the "per cent of control." In the tables we have given values of "100 minus per cent of control:"

$$\text{"100 minus per cent of control"} = 100 - 100\,\frac{M}{M_o} = 100\left(1 - \frac{M}{M_o}\right)$$

This gives a value which increases with the lactenic activity. Boiled whey has a value of zero and whey (from milk heated at 58°C. for 20 minutes) has a value of 100.

To obtain the activity of a preparation, its data are plotted as in Figs. 1, 2, or 3 together with the data of untreated whey. The length of a horizontal line connecting the two curves gives the logarithm of the relative activity.

The effect of pH on lactenin is shown in Experiment 1.

Experiment 1.—To 10 cc. portions of whey various quantities of M HCl and M NaOH were added (as shown in Table I). The tubes were then incubated 1½ hours and the reaction adjusted to the original pH. Each lot was then distributed in small tubes and refrigerated overnight. The next morning the tubes were inoculated with the 16 hour broth culture and the plates prepared before incubation and at intervals thereafter. All plate cultures were incubated 24 hours when the colonies were counted.

The whey retained its activity over a relatively narrow range of acidity and alkalinity. Thus when the acidity was brought to pH

[3] We are indebted to Dr. M. Kunitz of The Rockefeller Institute for Medical Research for helpful suggestions in regard to this method of measurement.

3.93, a point, it should be said, below that associated with the bacterial souring of milk, lactenin was not appreciably inactivated. A lower pH resulted in injury, and at pH values still lower than those shown in Table I it was completely inactivated. Similarly, although a reaction of pH 10.24 failed to decrease the inhibitory activity, when stronger alkali was added the inhibitory property was lost.

TABLE I

The Effect of Acidity and Alkalinity on the Lactenic Activity of Whey

Indicated amounts of M HCl or M NaOH were added to 10 cc. samples of whey, which were then allowed to stand 1.5 hours, neutralized and tested.

Volume HCl	pH before neutralization	Streptococci per cc. after					
		0 hrs.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	48 hrs.
cc.							
0	6.8	384	422	320	320	2	0
0.15	4.9	371	282	323	307	5	0
0.2	4.57	323	307	371	294	8	0
0.25	4.26	409	358	320	371	13	0
0.3	3.93	345	512	397	435	17	0
0.4	3.26	422	550	473	6,528	∞	∞
Control*		499	5,504	86,400	∞	∞	
Volume NaOH							
0	6.54	704	716	589	412	2	0
0.15	9.5	742	537	461	448	0	0
0.2	9.67	691	627	525	576	0	0
0.25	10.0	665	665	768	576	24	0
0.3	10.33	742	614	627	576	0	0
0.4	10.58	768	883	934	806	25	1
Control*		1,024	15,550	43,000	∞	∞	

* For a control, portions from all tubes were combined and boiled.

The Effect of Dialysis.—As stated in the introduction, whey contains considerable sugar and salts in addition to 0.4–0.5 per cent protein. It will be shown in Experiment 2 that the sugar and salts may be completely removed by dialysis, leaving the protein in the collodion bags together with essentially all the lactenin.

Experiment 2.—Whey from the mixed milk of a number of cows was prepared as usual. Measured portions were placed in tightly stoppered sterile collodion

sacs and dialyzed for 24 hours with flowing distilled water at room temperature in an apparatus which has been previously described.[4] The tubes were mechanically agitated throughout this period. Ordinary tests failed to show the presence of salts or carbohydrates in the dialyzed whey; a portion of the protein was precipitated but considerable still remained in solution. During dialysis the acidity was increased from pH 6.6 to pH 6.2. As a control a portion of the original whey was kept in the room during the dialysis. Both lots were then filtered through Grade V Berkefeld filter and tested for their inhibitory property.

That considerable activity was still retained after dialysis is brought out in Table II.

TABLE II

Effect of Dialysis on the Lactenic Activity of Whey

Product	Number of colonies per cc. after					
	0 hrs.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	48 hrs.
Whey	802	488	192	77	38	896
Boiled whey	1,600	23,360	∞	∞	∞	
Dialyzed whey	640	960	2,988	1,408	6,848	1,024
Boiled and dialyzed whey	1,088	4,544	14,400	∞	∞	∞

When the effects were measured by adding various quantities of both whey and the dialyzed product to Petri dishes little difference in inhibitory quality could be shown. When 1 cc. portions of each product were added growth was completely inhibited. When smaller quantities were used the colonies became progressively larger and the hemolytic zones more fully developed. During dialysis a small precipitate was formed which was slightly inhibitory.

Proteolysis, Dialysis and Concentration.—The solution left in the collodion bags was shown to be free from salts and sugar but contained the lactenin together with 0.4–0.5 per cent protein. Attempts to concentrate this dialyzed whey by the usual means failed, and attempts to fractionate the protein by precipitation with alcohol, ammonium sulfate, etc., resulted in inactivation.

It was now found that a part of the remaining protein could be removed by the action of trypsin followed by dialysis. In Experiment

[4] Kunitz, M., and Simms, H. S., *J. Gen. Physiol.*, 1928, **11**, 641.

3 whey was treated in this manner. The dialysis was performed in the concentrating dialyzer described in the preceding article,[5] and was concentrated nearly to dryness. This concentrated solution, when diluted back to its original volume, contained only 0.12 per cent protein but was essentially as active as the original whey, and when tested in the concentrated form it completely prevented growth of the scarlet fever streptococcus.

TABLE III A

The Lactenic Activity of Whey and of the Concentrated Products of Whey as Measured by the Hemolytic Zones of the Scarlet Fever Streptococcus

Dimensions represent longest diameter of the hemolytic zones in mm. Each value is the average of five readings which differ by less than 0.1 mm. from the average.

Volume per plate culture*	1.00 cc.	0.50 cc.	0.25 cc.	0.125 cc.	0.063 cc.	0.031 cc.	Relative activity: In conc. used	Relative activity: In original volume
Boiled whey (control) average (mm.)		2.4					0	0
Whey average (mm.)	0.52	0.94	1.36	2.12				
100—% of control	78	61	43	9			1.0	1.0
Concentrated digested dialyzed whey (1/20 of original volume) average (mm.)			0*	0*	0*	0.52		
100—% of control			100	100	100	78	32	1.6
Concentrated digested dialyzed whey rediluted to original volume average (mm.)	0.5	1.16	1.6	2.1				
100—% of control	79	52	33	9			0.9	0.9
Residue from 1 cc. digested dialyzed concentrate in 4 cc. NaCl + $NaHCO_3$ (1/5 of original volume) average (mm).	0.98	2.2						
100—% of control	59	8					0.3	0.06

* Zero size means not visible by 40 diameters magnification.

[5] Simms, H. S., *J. Exp. Med.*, 1930, **51**, 319.

Experiment 3.—A trypsin solution was made by suspending 5 grams of Fairchild's trypsin in 100 cc. of sterile NaCl solution and passing the centrifuged supernatant fluid through a Berkefeld V candle. Whey, prepared from fresh milk which had been heated at 58°C. for 20 minutes, was treated with this trypsin solution in the proportion of 1 cc. of trypsin solution for each 20 cc. of whey. The whey-trypsin mixture was incubated 2 hours and placed in sterile collodion sacs, then dialyzed and concentrated in the apparatus described in the preceding article.[5] After 4 hours the dialyzed material was withdrawn from the sacs, pooled and measured. It was found that the digested material had been concentrated to 1/20 of its original volume.[6] It was chilled overnight in the refrigerator and a slight precipitate was centrifuged off.

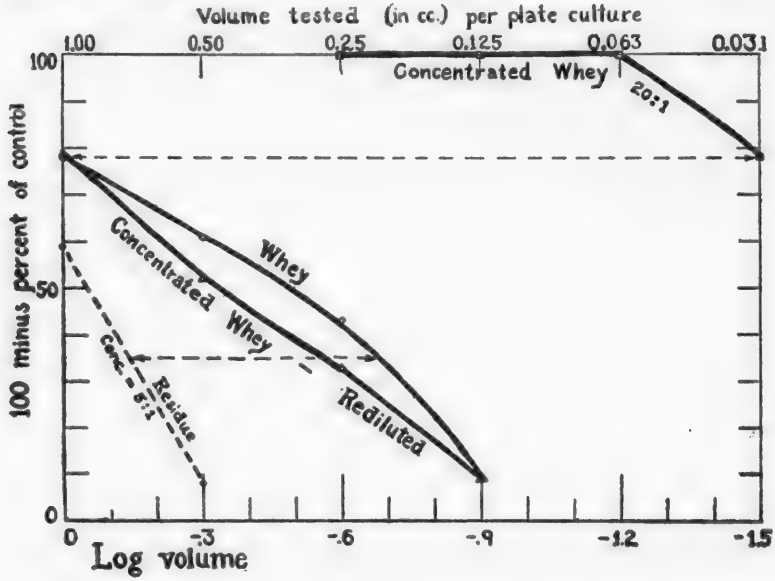

FIG. 1. The lactenic activity of whey and the concentrated products of hydrolyzed whey, as measured by the hemolytic zones of the scarlet fever streptococcus. The length of the horizontal dotted lines measure the logarithms of the relative activities.

[6] Concentration may be continued to complete dryness by this method, but it is best to remove the material when about 3 cc. is left in each bag. This avoids unnecessary loss.

A portion of the original whey which had been refrigerated during this period was tested as a control. Sterile salt solution was added to the control whey, in a volume proportionate to the trypsin used in the digested whey.

TABLE III B

The Effect of Concentrated Dialyzed Digested Whey on the Multiplication of the Scarlet Fever Streptococcus

Substance	Number of colonies per cc. after					
	0 hrs.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	48 hrs.
Whey	205	230	264	62		0
9.5 cc. boiled whey, + 0.5 cc. concentrated dialyzed digested whey	230	264	230	1,280	0	0
9 cc. boiled whey, + 1 cc. concentrate	230	264	51	26	0	0
9 cc. boiled whey, + 1 cc. concentrate boiled 5 minutes	294	2,816	17,280	∞	∞	
Boiled whey	320	4,548	∞	∞	∞	

Part A. Testing Materials.—Whey, concentrated material, and the residue centrifuged from the concentrated material were tested as to their effect on the hemolytic activity of the scarlet fever streptococcus. The residue was tested when dissolved in NaCl solution and a little $NaHCO_3$. The concentrated digested whey was tested both as such and also when diluted to its original volume.

The measurements given in Table III A are those of the longest diameter of the zone of hemolysis about the deep colonies of the scarlet fever streptococcus, as described above. The data are also plotted in Fig. 1.

Part B. The Effect of the Addition of Concentrated Material to Boiled Whey.—When the concentrated digested material is added to boiled whey in amounts sufficient to dilute it to its original concentration, or twice its original concentration, the inoculated streptococci fail to multiply and eventually die (as shown in Table III B). The boiling of whey destroys its lactenic activity so that growth begins soon after inoculation, but addition of the concentrated material prevents multiplication.

The Preparation of Active Dried Material.—The following experiment shows that the material of Experiment 3 may be completely desiccated with but slight decrease in activity. The material when partially dried is sometimes clear yellowish and vitrous, like dried varnish, but when completely dried is easily crumbled to a fine powder.

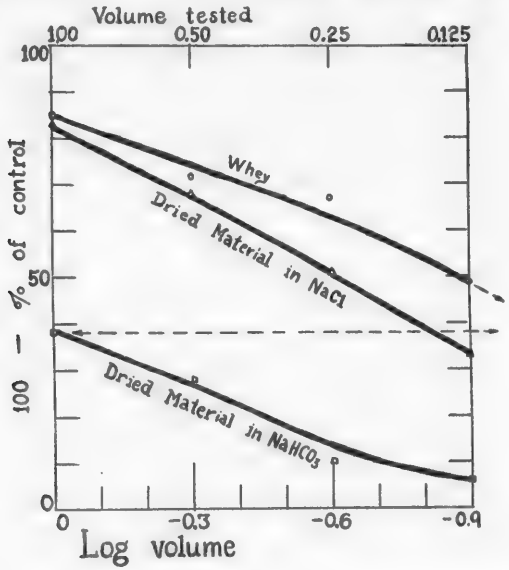

FIG. 2. The lactenic activity of dried dialyzed hydrolyzed whey when diluted to its original volume.

Experiment 4.—A portion of the dialyzed digested material which had been concentrated in Experiment 3 to 1/20 of its original volume was dried in high vacum over $CaCl_3$. After 36 hours the dried material was divided into two portions and NaCl solution was added to one in an amount equivalent to the original volume of whey from which it had been obtained. The remainder was taken up in a proportional amount of NaCl solution containing N/10 $NaHCO_3$. After thorough mixing both lots were stored in the refrigerator for 18 hours and then tested as in the method described. The measurements of the longest dimension of the hemolytic area are given in Table IV A and plotted in Fig. 2.

There is no indication that the substance has been materially injured by drying and the other steps in the process, provided that it is

TABLE IV A

Lactenic Activity of Dried Dialyzed Whey When Diluted to Its Original Volume
(From longest diameters of hemolytic zones; see Table IIIA)

Volume per plate culture		1.00 cc.	0.50 cc.	0.25 cc.	0.13 cc.	Relative activity origina volume
Control	average (mm.)	2.76				
Whey*	average (mm.)	0.46	0.76	0.9	1.4	
	100—% of control	85	72	67	49	1.0
Dried material suspended in NaCl solution (pH 6.0)						
	average (mm.)	0.46	0.88	1.36	1.86	
	100—% of control	83	68	51	33	0.7
Dried material suspended in 0.1 M $NaHCO_3$ (pH 9.0)						
	average (mm.)	1.7	2.0	2.3	2.6	
	100—% of control	38	28	10	6	0.1

TABLE IV B

Lactenic Activity of Dried Material Kept 3 Months and Diluted as Indicated
(From longest diameters of hemolytic zones)

Volume per plate culture		1.00 cc.	0.50 cc.	0.25 cc.	0.13 cc.	Relative activity in origina volume
Control	average (mm.)	2.64				
Whey*	average (mm.)	0.42	0.62	0.82	1.14	
	100—% of control	84	76	69	57	
Dried material after 3 months, diluted to original volume						
	average (mm.)	0.54	1.12	1.96	2.14	
	100—% of control	80	58	26	20	(0.7)*
Residue from above solution suspended in 1/3 of original volume						
	average (mm.)	0.44	0.79	1.5	2.34	
	100—% of control	83	70	43	10	(0.3)*

* The whey in this experiment is fresh and therefore cannot be accurately compared with the 3 months old preparation. The fresh whey is included in this experiment as a control on the culture of streptococcus. The values of relative activity in the last column are therefore not quantitatively correct.

resuspended in NaCl solution. If, however, sufficient sodium bicarbonate is added to the salt solution so that the final reaction is pH 9.0, considerable destruction takes place during overnight refrigeration.

The Effect of Time.—Dried material prepared in the same manner and stored 3 months in the refrigerator and in the room, still retained much of its activity, as shown in Table IV B and Fig. 3.

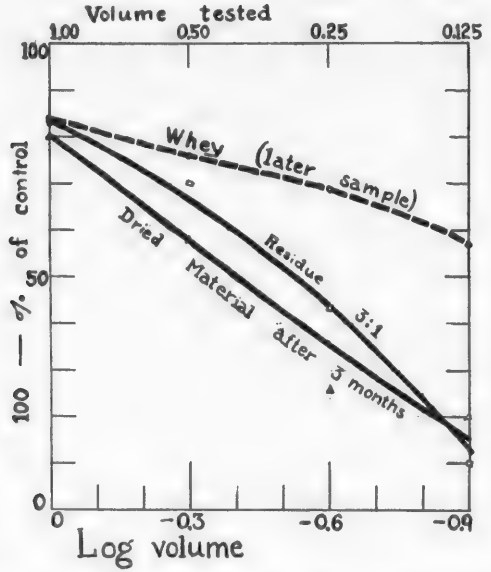

FIG. 3. The lactenic activity of the dried material after standing 3 months

Highly Active Material.—When sufficient salt solution was added to the dried material to bring it to the volume of original whey a portion failed to dissolve. This fraction from 12 cc. of whey was separated by centrifugation and suspended in 3 cc. of NaCl and 1 cc. of N/10 $NaHCO_3$ and tested. The results are included in Table IV and Fig. 3. It was found to have nearly one-half the activity of the dissolved portion. But it represents only one-eighth the dried weight. Hence this residue is 3 or 4 times as active as the dissolved portion on the basis of dry weight, or *about 200 times as active as skimmed milk*, on the same basis.

DISCUSSION

The experiments give us some information concerning the properties of lactenin. It is stable for 1½ hours at pH values ranging from 4 to 10. If the time of exposure be lengthened the material must be kept even closer to the neutral point. We have shown that lactenin is not associated with salts and carbohydrates but can be readily separated from them by dialysis. Lactenin appears to be associated with the proteins of whey, since agents which precipitate protein from whey also remove the lactenin, although lactenin is not removed with casein during rennet coagulation. Furthermore lactenin, like proteins, fails to pass through permeable collodion membranes. It and some of the whey proteins resist the digestive activity of trypsin, but the split products of the proteins hydrolyzed by trypsin are removed by dialysis without impairing lactenic activity. We have no evidence that lactenin is itself a protein.

TABLE V

Activity of Lactenin Material on a Fat-Free Dry Weight Basis

(Skimmed milk is taken as unity)

Material	Per cent solids	Relative activity per gram dry weight
Whole milk	13.6	1.0
(Fat removed by centrifuging)	−4.0**	
Skimmed milk	9.6	1.0
(Casein removed with rennet)	−3.1	
Whey	6.5*	1.5
(Salts and sugars dialyzed out)	−6.1	
Dialyzed whey	0.41*	23
(Proteins hydrolyzed and removed by dialysis)	−0.29	
(Protein added with trypsin)	+0.003	
Dialyzed hydrolyzed whey	0.123*	70
(Redissolved)	−0.109	56
Residue on redissolving	0.014*	205

* Dry weights determined.

** Normal value assumed.

In Table V will be found the dry weights of various lactenin materials. It will be seen that the dialyzed hydrolyzed whey contains

only 1.3 per cent of the original fat-free solids of milk. The tests indicate that this contains at least 90 per cent of the original activity of milk.[7] Hence it is 70 times as active as skimmed milk on a dry weight basis.

The material which has been digested, dialyzed and concentrated may be dried and stored for some time without appreciable loss of activity. When this material is dissolved it is about 56 times as active as the original milk, on a dry weight basis. A portion of the material fails to dissolve in physiological NaCl solution. This fraction is 205 times as active as the original milk, on a dry weight basis. The chemical nature of this very active material will be discussed in a future publication.

The method of measuring the size of hemolytic zones proved to be a reliable index of lactenic activity of substances which are not highly nutritive. It is possible to measure the effect of as little as 0.13 cc. of whey or other active products when mixed with 12.5 cc. of nutritive medium (veal infusion agar + horse blood) upon the development of the colonies and hemolytic zones of the scarlet fever streptococcus. With whey this means that its activity is shown in a final dilution of 1:100. The residue from the dried material is active with 0.005 mg. per cc. of plate culture. The relative activity of different materials is obtained by the horizontal distance between curves of data plotted as in Figs. 1 to 3.

SUMMARY

The bacterial growth inhibitory substance found in milk is called *lactenin* in this paper. It is stable for 1½ hours at pH 4 and at pH 10 and for longer periods in neutral solution. It is not associated with salts and carbohydrates and may be separated from them by dialysis.

Lactenin is removed by agents which precipitate the proteins of whey. Part of these proteins may be hydrolyzed by tryptic digestion and the resulting split products, together with the salts and sugar, may then be removed by dialysis without appreciable loss of lactenic activity.

[7] If we allow a 10 per cent loss of material in removing it from the bags, there is no appreciable loss in activity.

This dialysis may be performed in a concentrating dialyzer, under sterile conditions and at low temperature, thus reducing the solution to small volume. The material may then be completely desiccated and kept 3 months with practically no loss of activity. The residue, on treating this dried material with salt solution, is 200 times as active as the original milk, on a dry weight basis.

The size of hemolytic zones of the scarlet fever streptococcus grown on a medium containing lactenin is found to furnish a simple and reliable measure of lactenic activity.

CHEMOTHERAPEUTIC EQUILIBRIA

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(Received for publication, November 27, 1929)

The statement has been made (1), with reference to the apparent fruitlessness of the search for specific chemotherapeutic agents, that "the real difficulty lies in the necessarily opportunistic experimental method, and the lack of a rational scientific means of approach." The considerations presented in the present paper are sufficiently general to be valid regardless of the mechanism of chemotherapeutic action which may be eventually discovered; and it is hoped that they will suggest a tentative rational approach to such a study.

Bacteriochemical Equilibria

A large amount of work done *in vitro* has indicated that the reaction between the bacterial cell and some, at least, of the common chemotherapeutic agents is chemical in nature and indicates a behavior which can be predicted by the law of mass action (2).

The bacterium is pictured as an equilibrated amphoteric system of more than one component. Such systems have been shown to behave in many respects as simple ampholytes (3).

The belief that the reaction between the organism and the agents studied is chemical is founded on extensive studies of staining behavior, bacteriostatic behavior, and the effect of oxidizing agents and decolorizing agents on the former. The following series of equilibria may be thought of as establishing themselves in a system of bacteria and basic agent. For considerations of staining actions this agent will be a basic dye; for bacteriostatic considerations it may or may not be a dye. We will indicate the fact that the agent is basic by the type formula DOH, and that the bacterium is amphoteric by the formula HBOH.

1. $DOH \leftharpoons D^+ + OH^-$
2. a) $HBOH \rightleftharpoons H^+ + BOH^-$
   b) $HBOH \leftharpoons HB^+ + OH^-$
3. $D^+ + BOH^- \rightleftharpoons DBOH$
4. $DBOH + HOH \leftharpoons HBOH + D^+ + OH^-$

Maximum retention of stain, or maximum bacteriostatic effect, will be expected under conditions favoring maximum formation of the unionized dye-bacterial compound, DBOH (Equation 3). The longer arrows in the above set of equations indicate the directions in which the equilibria would shift due to an increase in alkalinity.*

For staining reactions we have the following predictions and verifications:

1. Increase in pH should increase the retaining power for dye. This fact is now generally recognized. At sufficiently high pH values even Gram negative organisms may appear to be Gram positive (2c).

2. The acidic strength of the organism should be a factor. Actually, determinations of the isoelectric ranges of a number of organisms showed that these ranges in the case of Gram positive organisms occurred at a significantly higher acidity than those characteristic of the Gram negative organisms as a class (2f) (4).

3. Any process which tends to increase the acidic strength of the same organism should increase its inherent retaining power for dye. Actually it has been shown that by oxidation the retaining power of any organism for basic dye is increased. By sufficiently vigorous oxidation any Gram negative organism can be made to appear Gram positive. Subsequent reduction tends to reverse this effect (2c).

4. If these reactions represent the partial attainment of an equilibrium condition, they should be easily reversible. Actually this is demonstrated by the ease of decolorizing any non-capsulated organism with an acid decolorizer.

5. If the type of bond between organism and dye is chemical, then the general nature of the decolorizer used should determine its functioning as a decolorizer apart from the mere solubility of the dye in it. In this connection the authors have shown (2j) (2k) that basic decolorizers show abnormally high decolorizing power toward smears stained with acid dyes, and acid decolorizers show the same abnormal behavior toward smears stained with basic dye. It should be borne in mind that this effect is independent of any pH effect on decolorization and depends only on the chemical nature of the decolorizers. For example aniline would be a representative basic decolorizer while aldehydes would be acidic in nature.

In the case of acid dyes what may be thought of as a reverse behavior to that toward basic dyes has been in every case found to exist. Such could be predicted from an analogous set of equations.

Analogously we would have for bacteriostatic reactions, the following predictions and verifications:

1. Increase in alkalinity, which shifts the equilibria toward the formation of com-

* Equation 4 takes account of the fact that the effect of increasing alkalinity on the amphoteric cell is much greater than on the purely basic dye through the ordinary pH range where such studies are of importance, even though the respective ionization constants may not be so widely different. In this pH range the dyes, at least, would be almost wholly in the form of salts and thus, for comparison of effect only, the dye is represented as being highly ionized compared to the protein constituent.

pounds represented by the type formula DBOH in the equations, should increase the effective dilution of a basic bacteriostat. In the case of dyes this is, as is now well known, the case where such increase in alkalinity is not sufficient to precipitate out the agent or to alter its chemical nature (2g) (2h).

2. These bacteriostatic effects should also be reversible. That is, as an example, a concentration of a dye which would just inhibit growth at a certain pH should fail to inhibit it at a lower pH. This has been definitely shown by the authors (2g). Such a reversal can also be brought about in other ways. Any method of dissociating the molecule represented by DBOH by removing the basic D^+ ion will be effective. This is shown by the work of Englehardt (5), who removed mercuric ion by sulfide precipitation, and found that staphylococci which had been treated for 72 hours in a 1% bichloride solution would, after the removal of the mercuric ion, grow out. Similarly apropos in this connection are the results obtained by Churchman (6) on injection of stained *Bacillus anthracis*, which were found to remain apparently innocuous for periods from 10 to 20 times longer than those required by unstained organisms to produce death, but which finally suddenly revived with fatal results. Here, of course, we cannot put our finger on the chemical mechanism by which the positive dye ion was removed from action.

3. We should expect increase in basic strength of dye to increase its effectiveness. As the authors have pointed out (2e), all available data indicate such to be the case, but such data are meager. This factor cannot be generally treated because different classes of agents show effects which are of an entirely different order of magnitude. Also through the pH range usually studied most of them will be in salt form and but slightly hydrolyzed.

4. The presence of protein or other matter of a similar nature which chemically resembles bacteria should decrease the effectiveness of any agent by binding a portion of it in an ineffective combination. This has been shown to be so by Graham-Smith (7), Winslow and Dolloff (8), Wels (9), and others.

5. If mass action tends to govern bacteriostatic effects, the amount of bacterial inoculum should be a factor. That this is so has been qualitatively demonstrated by many, and semi-quantitatively shown by Gay and Beckwith (10), Browning and Gulbransen (11) and Graham-Smith (7), among others. While this last mentioned phenomenon has received other explanations, it is at least completely in harmony with the point of view outlined above.

6. Finally we may expect the acidic strength of the bacteria to be a factor. The well known fact that basic dyes are more effective against Gram positive than against Gram negative organisms, coupled with our findings on the isoelectric ranges of these organisms showing the Gram positive ones to be more strongly acidic, constitutes the experimental verification of this prediction.

The probability of the general correctness of the above outlined point of view is strengthened by the analogous behavior of organisms toward acidic substances, which behavior can also be successfully predicted from an analogous set of equations, as well as by the general similarity of all such behavior to the simple protein chemistry.

Stoichiometrical Relationships

Whatever be the mechanism of internal therapy, there seems to be evidence *in vitro* of some action between agents and organism which follows ordinary stoichiometrical laws. It is therefore of interest and importance, in the first place, to inquire whether the quantities of therapeutic agent which have been suggested from time to time are stoichiometrically sufficient. Recently the dyes gentian violet and mercurochrome have been used to a considerable extent in blood therapy. Take the former as an example.

A concentration of 1:10,000 in the blood stream has been found safe and has been used. Taking the equivalent weight of the dye as about 400, we have, at this

TABLE I

Showing the Effect of Blood Constituents on the Effective Concentration of Dye against Bacteria in the Blood

Equiv. dye ion orig.	Approx. equiv. protein anion	Approx. ionization const. for dye-protein salt*	Equiv. dye ion. final	Equiv. bacterial protein	Approx. ratio $\frac{\text{Dye equiv.}}{\text{Bact. equiv.}}$
1	20 (plasma)	0.0005	0.01	0.00004	250.
1	35 (whole blood)	0.0005	0.006	0.00004	150.

* This magnitude is obtained from experiments of the type described in section on "Abnormal ionization equilibria."

concentration, one equivalent of dye to about 4,000,000 parts of blood. In the blood we have, besides the bacteria which are being combated, the blood proteins to consider, especially those of the plasma which, at the pH of blood, are on the alkaline side of their isoelectric points. These proteins are in large excess compared with the bacteria. If we assume that they have characteristics not too dissimilar from those simple vegetable proteins so exhaustively investigated by Hoffman and Gortner (12) we can get a fair idea of the order of magnitude of the concentration of protein anion. In Table I are recorded results of such calculations. We have assumed the composition of the blood as given by Mathews (13). A preliminary determination of the ionization constant of the dye base of gentian violet indicated that, since it is added as the chloride, the dye will be present practically entirely as cation until bound by bacteria or some component of the blood. The number of equivalents of fibrinogen can be assumed by reading directly from the curves of Hoffman and Gortner for fibrin; while the number of equivalents of the other proteins are assumed to be about the same as they would be if they consisted of the

prolamines studied by these workers, twelve of which showed almost identical behavior at blood pH. The various quantities in Table I are calculated on the basis of one equivalent of dye ion, i.e. for 4,000,000 parts of blood.

The bacterial equivalents are calculated on the assumption of a count of 10,000 per cc. (2e). The final ratios, then, represent lower limiting conditions. Work on the staining behavior of blood cells, both sheep and human (15) indicates that the cells as a whole have an isoelectric point at a pH of about 6 to 6.4 so that at blood pH they are just beginning to show an appreciable affinity for basic dye. It is therefore more than probable that they do not affect the effective dye concentration to nearly the extent assumed in the values given in Table I. Moreover a bacterial count of 10,000 per cc. seems to be also a fairly limiting case. Under actual conditions the effective dye-bacterial ratio would probably be nearer many hundred than the values given.

These values indicate that, even in blood where it might be expected that dye would be completely combined with blood proteins which are somewhat in excess, a total dye concentration found clinically safe to use, 1:10,000, may be expected to furnish a bacteriostatic value corresponding to an *in vitro* concentration of 1:1,000,000 or 1:2,000,000 (i.e. 0.01 to 0.006 parts in 10,000 as in column 4 of Table I). These latter concentrations are not only in large stoichiometric excess over bacterial equivalents but are concentrations found bacteriostatic *in vitro* for many strains, especially of Gram positive organisms.

Recently some work reported by Hirschfelder and Wright (14) indicates that a concentration of crystal violet 1:20,000 in the presence of 1% albumin showed the same antiseptic power as a 1:35,000 solution in the absence of protein. No mention is made of buffering the system though the results were obtained by a process in which CO_2 was liberated. This, in an unbuffered system, might significantly affect the pH, which has been shown (2g) to greatly influence the effectiveness of dyes, and one cannot judge critically the conclusions of the authors that even adsorbed dye has some antiseptic value. This conclusion was reached on the basis of calculations from adsorption data which indicated that this 1:20,000 dye solution should have had the same antiseptic power in 1% albumin as a 1:125,000 solution in water. Details of measuring adsorption are not given so that one cannot judge the applicability of these results to blood conditions, but the work would tend to confirm the conclusions reached from the above calculations.

Time of Establishing Dye-Protein Equilibrium

A puzzling feature regarding the action of therapeutic agents, especially in the blood, which has often been brought up, is the apparent rapid disappearance of the agent. In this connection the results of two experiments are presented.

Table II embodies the results of some time experiments on the establishment of practical equilibrium between finely cut dry gelatin slabs and solutions of gentian

violet. Naturally the time values may be in error by 50% or more on such short times, but the results are suggestive. In obtaining these results definite amounts of gelatin were shaken vigorously with definite volumes of gentian violet solution of known concentration and controlled alkalinity, for definite periods of time. A portion of the dye was by this means bound by the gelatin and removed from

TABLE II

Showing the Approximate Length of Time Required for Equilibrium between Gentian Violet and Gelatin

cc. Dye soln.	Grams gelatin	cc. N/2 NaOH added	Time shaken (sec.)	Orig. dye concn. p.p.m.	Final dye concn. p.p.m.	Final dye concn. p.p.m. (blank)
10	0.08	—	60	10	8.0	9.0
10	0.08	0.20	10	10	5.0	
10	0.08	0.35	10	10	2.0	7.5
10	0.10	0.20	10	10	5.0	9.5
	(*same tube*)		25		3.0	
			40		3.0	
			60		3.0	8.5

TABLE IIa

10	0.10	—	20	10	7.0	
10	0.10	0.04	12	10	6.0	
10	0.10	0.20	10	10	4.0	9.0
10	0.10	0.40	10	10	3.5	9.0
10	0.10	—	20	5	3.5	
10	0.10	0.04	12	5	3.0	
10	0.10	0.20	10	5	1.5	
10	0.10	0.40	10	5	1.0	4.5

(Longer shaking in these latter cases made no difference in the results.)

Results in Table II were obtained using a solution of gentian violet in water; those in Table IIa were obtained using a solution of the dye in M/20 disodium phosphate.

solution, and the intensity of the color remaining in the supernatant liquid was compared with standard solutions of the dye.

This experiment indicates that, if the above pictured mechanism of bacteriostasis is true, the time required for a bacteriostat to become effective is conditioned largely by speed of mixing with an infected fluid and perhaps of penetration into organisms. The actual bacteriostatic equilibrium seems to establish itself very rapidly when mutual contact of dye with the particular component with which it may combine is obtained.

This simple experiment indicates that, when there are no disturbing factors and when agitation is vigorous, a primary equilibrium is very rapidly reached between protein and dye. Although, in the case of blood therapy, the method of administration of the agent would seem to lead to a rather rapid and thorough mixing, one may expect in such a medium, where blood proteins are so enormously in excess of bacterial protein, that the time required by the agent for action may be considerably increased.

It should be pointed out that the time required for the establishment of this type of equilibrium should not be confused with the times, reported through the literature, required for "killing" an organism. An agent in the blood may be effectively holding in check the normal development of an organism, yet when a sample of this same blood is plated out it may appear by no means sterile. This may be due as we have seen above to an alteration of any of the factors which have been shown to reverse a bacteriostatic equilibrium.

Alteration of Therapeutic Agent in the Blood

The other consideration we wish to present in connection with the apparent rapid disappearance of such an agent from the blood is that this apparent disappearance may not mean that the blood no longer retains some effect. It seems to be necessary for certain agents, notably certain pentavalent arsenic preparations, to be reduced by the body tissues to trivalent arsenic before they produce the desired effect. It was on such a basis that Ehrlich accounted for the clinical trypanocidal effectiveness of atoxyl, which, *in vitro*, is ineffective against these organisms even at a concentration of 5 per cent. On the other hand the trivalent arsenic in the form of the oxide is immediately trypanocidal at a concentration of 1:100,000.

Gentian violet is rather easily decolorized with either nascent hydrogen or hydrogen peroxide. The following experiments were performed.

Solutions of the colorless products were prepared using both methods. For the first method zinc and hydrochloric acid were employed, and the bulk of the zinc was removed by precipitation with ammonia. The resulting solution was tested for bacteriostatic action. Controls of ordinary gentian violet as well as of the zinc salt, prepared from zinc and acid as in the reduction mixture, were run. The bac-

teriostatic activity of a dye solution decolorized by hydrogen peroxide and in which the excess peroxide had been decomposed was also tested. Results are presented in Table III. They are, of course, preliminary in nature, but they seem to have a significance in themselves aside from their bearing on a rational picturization of a mechanism for blood therapy, especially in the case of agents which seem to rapidly disappear. It may be pointed out that reduction by zinc and acid does not

TABLE IIIa

Showing the Bacteriostatic Effect of Decolorized Gentian Violet

Organism	pH	Zn soln. control	G. V. red. by nascent hydrogen sample #1	G. V. red. by nascent hydrogen sample #2	Normal G. V. control	Dilution of agent
B. coli.....	5.5	+ + +	− − −	− − −	− − −	1:20,000
Strain 1	6.5	+ + +	− − −	− − −	− − −	1:20,000
B. coli.....	5.5	+ + +	− − −	− − −	− − −	1:20,000
Strain 2	6.5	+ + +	− − −	− − −	− − −	1:20,000

TABLE IIIb

Organism	Dilution of agent	pH	G. V. red. by peroxide	G. V. red. by nascent hydrogen	Normal G. V control
B. coli.....	1:10,000	5.5 to 6.0	− −	− −	——
B. coli.....	1:20,000	5.5 to 6.0	− +	+ +	− +

TABLE IIIc

B. coli.....	1:10,000	5.5 to 6.0	− −	− −	− −
B. coli.....	1:20,000	5.5 to 6.0	——	——	− −

give the colorless product quite a fair chance in this test, since the gelatinous zinc hydroxide, which comes down in considerable quantities, is bound to carry down with it a considerable amount of active material which is thus removed.

The medium was ordinarily plain nutrient broth adjusted to a certain pH and containing a known amount of bacteriostat. In Table IIIa are given growth results in two samples of the dye decolorized with nascent hydrogen for 24, 48 and 72 hour incubation periods. (In this particular experiment the medium was 1%

lactose broth.) In Tables IIIb and IIIc results are given for growth in one sample of dye decolorized by nascent hydrogen and in one decolorized by hydrogen peroxide. Results are for 24 and 48 hour incubation periods. A slow precipitation of zinc hydroxide, brought about by pH adjustment, seemed to carry down nutrient material and, presumably, active agent as well. Therefore a trial was made on tubes in which the precipitation was hastened as much as possible and the precipitate centrifuged out. 24 and 48 hour growth results are given in Table IIIc, otherwise this table is similar to IIIb.

We are not, of course, suggesting that gentian violet is decolorized by these mechanisms in the blood, but it is of interest to note that disappearance of color in the case of such a reagent does not in itself mean disappearance of therapeutic effect.

At first sight these results may seem in disagreement with the statement of Dubos (16) who, from a study of the bacteriostatic effect of certain dyes which form reversible oxidation-reduction systems, finds that "the dyes are not toxic in the reduced form." His results are not comparable with those reported here, however, since he is working with reversible systems, systems which are reduced by the medium rapidly even in concentrations as high as about 1:5,000 and therefore systems which would tend to poise the medium at a certain oxidation potential. This poising effect he points out as a factor in the mechanism of dye bacteriostasis. There is no doubt in the minds of the present authors as to the validity of this claim where it can be shown that the bacteriostatic systems are effective as poising agents. In this paper bases for the claim of another factor are given. The relative importance of the two factors will depend on the particular systems. The most direct evidence of this other factor, which may be thought of as covalent salt formation between bacteriostatic agent and some constituent of the organism, will be found below.

Flocculation Equilibria

It may seem at first sight a far cry from the above specifically chemical equilibria to the phenomenon of flocculation, with its possible relation to agglutination, which we usually consider as a surface phenomenon of physical nature. Results given in Table IV are typical. From such results as these it is indicated that flocculation behavior is influenced by essentially the same factors and in essentially the same way as is staining behavior.

Using care to differentiate between so-called acid agglutination and flocculation produced by the agent under observation, the following facts were ascertained:

TABLE IV

Showing the Effect of Dye Concentration and of pH on the Time of Flocculation of the Gram Negative B. coli and the Gram Positive B. cereus by Means of Basic and of Acid Dye

Floc. time (min.)	Basic fuchsin pH (approx.) 1	2	3	4	5	6	Floc. time (min.)	Acid fuchsin pH (approx.) 1	2	3	4	5	6
Organism—*B. coli*													
Dye concentration 1:200													
1	−	−	−	−	+	+	1	±	±	−	−	−	−
3	−	−	±	+	+	+	5	±	±	−	−	−	−
4	−	±	±	+	+	+	8	+	±	−	−	−	−
17	±	+	+	+	+	+	44	+	+	±	−	−	−
Dye concentration 1:250													
1	−	−	−	−	±	±	7	±	±	−	−	−	−
3	−	−	−	±	+	+	42	+	±	±	−	−	−
7	−	−	±	+	+	+							
22	±	+	+	+	+	+							
Organism—*B. cereus*													
Dye concentration 1:200													
0.5	−	−	−	−	+	+	2	±	±	−	−	−	−
2	−	−	+	+	+	+	11	+	+	±	−	−	−
3	−	±	+	+	+	+	19	+	+	±	−	−	−
4	±	+	+	+	+	+	60	+	+	+	±	−	−
Dye concentration 1:250													
1.0	−	−	−	−	±	+	1	±	±	−	−	−	−
1.5	−	−	−	±	+	+	7	+	±	−	−	−	−
2.0	−	−	±	+	+	+	17	+	±	±	−	−	−
2.5	−	±	+	+	+	+	60	+	+	±	−	−	−
6.0	+	+	+	+	+	+							

1. The basic dyes, gentian violet and fuchsin, are more effective in flocculating both the Gram negative *B. coli* and the Gram positive

B. cereus at higher than at lower pH values. That is, at constant dye concentration the flocculation is more rapid, while to attain a certain flocculation speed less dye is necessary, at high than at low pH values.

2. The reverse is true in the case of acid fuchsin. It should be pointed out in connection with material presented under "Abnormal ionization equilibria" that neither brucine nor nicotine cause flocculation at concentrations comparable to those of the dyes mentioned above. The experiments were made repeatedly, checking the macroscopic readings by microscopic examination.

If we picture the cell membrane, as suggested by Bayliss (17), as a variable structure in equilibrium with the changing states of the cell, the above results are easily brought into harmony with the general picture.

Any material within the cell which lowers the surface energy will accumulate at the surface. It has been shown (18) (19) (20) that alteration of the surface tension of media through wide ranges has no apparent effect on the viability of many bacteria. This means that the bacterial cell possesses a mechanism for adjusting its surface tension in response to changes in the surface tension of the medium so as to bring about a fairly constant interfacial tension between its surface and the medium. The obvious mechanism is a labile distribution equilibrium, between the surface and the interior of the cell, of some surface tension depressant such as a lipin or even a protein constituent. Addition of a reagent which possesses the power to bind such a substance will of course shift any surface equilibrium with the result that the interfacial tension between organism and medium may increase. The tendency therefore will be to decrease total surface and flocculation will result. Such binding agents are the dyes in the above experiments, their binding power having been previously shown to vary with pH in the manner necessary to explain the flocculation results.

It may be suggested that change in pH and addition of dyes may alter the surface tension of the medium in a way to cause flocculation. Measurements of surface tension of nutrient broth and of broth containing as high as 1 per cent dye and with pH varying between 2.5 and 8 gave a variation of only about 5 dynes, however, so that some other explanation must be sought than this.

Abnormal Ionization Equilibria

The question may well arise why, if we imagine the action of therapeutic agents to be pictured as taking place according to the set of equilibria formulated above, there is such a wide difference between the inhibiting dilutions of different groups of agents which seem to belong to the same general chemical type. More specifically the question may be put why such substances as the triphenylmethane dyes, for example, inhibit bacterial growth at effective concentrations enormously less than those at which other basic substances, whose basic strength may be as great or greater, are found to be effective. This question has led one of the authors to an investigation of equilibria of the type represented by equation number 3 above, namely the ionization of the compound represented by the formula DBOH. Obviously if this compound is highly ionized enormous excess quantities of D^+ ion would be necessary for it to be formed in appreciable amount.

To determine whether different types of basic substances behave in a significantly different manner with regard to the ionization of their protein salts, conductivity measurements were made on solutions of certain bases alone, on a solution of protein alone, and on mixtures of the two. Results are given in Table V.

For the protein a 1% gelatin solution adjusted to a given pH by means of NaOH was used. For the bases, potassium, as the chloride, nicotine as the free base and gentian violet as the chloride were employed at concentrations equivalent to that of the gelatin taking its equivalent weight toward bases as 3300 (21).

In the cases of both nicotine and potassium the measured conductivity of the mixture agrees with that calculated on the assumption that none of the ionic species present tend to form undissociated molecules. In the case of the dye-protein system, however, there is a significant "loss of conductivity" when the two are mixed. A considerable portion of the dye or protein ion disappears, and this can be most easily pictured as being due to the fact that we are here dealing with a salt type of compound which is only slightly ionized, analogous in its behaviour to such inorganic salts as lead acetate, mercury salts, and others, which, compared with the general run of inorganic salts, are only slightly ionized.

The results of this experiment offer an explanation for the difference in flocculation behavior between the alkaloids and dyes noted in the above section. In the same way they offer an explanation for the

findings (22) that nicotine, even at a concentration of 1:2,000, does not seem to inhibit the growth of either Gram positive or Gram negative organisms. The dye in each case actually seems to bind, in unionized combination, some protein-like constituent of the bacterial

TABLE V

Showing the Effect of Mixing Solutions of Protein Anion with Various Cations on the Conductivities of These Ions

Solution	Temp.	pH	Conductivity x 10^6 (measured)	Conductivity x 10^6 (calculated)	% Difference
A. Ionization of potassium proteinate					
KCl	23.5	unbuffered	108.6		
gelatin	23.5	7.65	38.4		
1:1 mixture	23.5	7.6	75.1	73.5	2.
B. Ionization of nicotine proteinate					
nicotine	24.9	9.5	13.5		
gelatin	24.9	9.4	39.0		
1:1 mixture	24.9	9.35	27.1	26.3	3.
C. Ionization of gentian violet proteinate					
gentian violet	23.6	7.25	27.0		
gelatin	23.6	7.3	38.4		
1:1 mixture	23.6	7.1	23.0	32.7	−30.
1 gel: 2 dye	23.6	7.15	18.5	34.6	−46.

The values in column 4, Table V, are the conductivities of the ions under consideration and not the total measured conductivities. KCl is corrected for the Cl ion, gelatin is corrected for the conductivity of the Na ion added in pH adjustment, etc. These corrections do not in any way affect the argument, though they do affect the magnitudes in the last column. If we take the total conductivities we find that for nicotine and potassium the observed conductivities for the mixture do not differ from the calculated values by more than a fraction of 1%. The decrease in the case of the dye in this way amounts to 13 to 18%. Since this decrease is not distributed among all of the ionic species present in the mixture but is practically confined to the ones discussed, a more definite idea of its magnitude can be had from the corrected data as given. The last system, namely gelatin and dye, has received fairly extensive study and results in detail are to be published elsewhere.

cell, in the one case shifting the surface equilibrium and causing flocculation, and in the other case interfering with normal cell development; whereas the alkaloid, not seeming to form such a type of linkage, is ineffective in both cases.

Ionic Displacement Reactions

It may be suggested that the above conductivity results are explicable on the basis of the mutual flocculation of oppositely charged colloidal particles and has no connection with any type of salt formation. In answer to such a suggestion the following experimental results are presented.

TABLE VI

Showing the Liberation of H-Ion When Gelatin Is Mixed with Dye Cation, and of OH-Ion When It Is Mixed with Dye Anion

	Crystal violet		Acid fuchsin	
	pH	$-\Delta$(pH)	pH	Δ(pH)
dye	3.92		3.13	
gelatin	3.91		3.13	
1:1 mixture	3.83	0.08	3.32	0.19
dye	4.64		3.75	
gelatin	4.64		3.75	
1:1 mixture	4.29	0.35	4.10	0.35
dye	5.64		4.60	
gelatin	5.64		4.62	
1:1 mixture	5.32	0.32	4.73	0.11

Consider an amphoteric substance represented by the type formula HBOH, which can exist in solution not only in the form of the neutral compound but also either as cation or anion depending on pH. Let it react with a basic dye ion and there are the following possibilities:

$$D^+ + BOH^- = DBOH$$

and

$$D^+ + HBOH = DBOH + H^+$$

If these equations represent the types of reaction taking place between dye and protein, there ought to be conditions, if the compound DBOH is sufficiently stable, such that H-ion is liberated when dye ion and protein in unionized form are mixed.

In an analogous manner there ought to be conditions under which, when an acid dye ion is substituted for the basic dye ion, OH-ion should be liberated. This can be easily tested out by adjusting dye solutions and protein to the same pH and noting any change on mixing (23). A large number of such experiments have been carried out using various proteins and various dyes. Typical results are given in Table VI, using gelatin as a protein, crystal violet as a basic dye and acid fuchsin as an acid dye. The gelatin was a 1% solution and the crystal violet was used as a solution of 1.5 grams per liter. The other dye solution contained 3 grams per liter.

Here the basic dye causes a decrease in pH corresponding to a liberation of H-ion, and the acid dye causes the opposite effect.

Is it possible that types of therapeutic specificity will be found to be somehow connected with the property of forming such un-ionized compounds with a cell constituent? This is at least a point worthy of consideration.

SUMMARY

1. The general adequacy of the bacteriostatic mechanism for the action of dyes which postulates a mass law equilibrium between bacteriostat and organism, which latter is pictured chemically as an ampholyte, is discussed.

2. It is shown that, even in blood, where, with safe concentrations of dye, there seems to be a significant excess of protein over dye, the stoichiometric excess of dye required by the above mechanism is available.

3. Experiments are presented indicating that the time required for such a bacteriostat to act is very short, being probably conditioned largely by speed of mixing or of penetration.

4. Apparent disappearance of dye from blood stream need not mean that the blood has lost bacteriostatic value.

5. Data are presented indicating that the behavior of dyes in causing flocculation of organisms is affected by the same factors and in the same way as in inhibiting growth.

6. Direct evidence of ionic combination between dye ion and protein ion is presented by noting conductivity decrease when the two ions are mixed, and also noting the displacement of H-ion from un-ionized protein by dye cation, or of OH-ion by dye anion.

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